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**Studies on the Influence of
Sex Hormones on Glucose Tolerance
and Insulin Secretion in the Rat**

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SUMMARY

This thesis examines certain aspects of the general hypothesis that 'sex hormones participate in the physiological regulation of carbohydrate metabolism!.

Studies in female rats at different stages of the oestrous cycle, following ovariectomy and during advancing age indicate that enhanced sexual endocrine function is associated with an improvement of glucose tolerance and an elevation of plasma insulin levels. This interpretation is substantiated by observations that treatment of ovariectomized females with oestradiol, progesterone and a preparation of gonadotrophins produces similar alterations of glucose metabolism and the circulating insulin pool. Evidence is presented that oestradiol and progesterone induce these changes within six hours, and that progesterone, but not oestradiol may directly promote pancreatic insulin secretion within this period.

Testosterone does not appear to exert a significant influence on glucose tolerance in male rats, although there is a tendency for this hormone to reduce plasma insulin levels.

Characteristic sex differences of glucose tolerance and circulating insulin levels have been detected and their possible relationship with the prevailing sex hormone environment is discussed.

The mechanisms through which sex hormones may effect glucose tolerance and insulin levels, and the implications of

these processes are considered with respect to their importance during different reproductive states in the female.

PREFACE

Carbohydrate metabolism is a ubiquitous function among living cells. It ranks as an integral component of intermediary metabolism and as such it is subject to the complex regulatory network that co-ordinates this diverse area. In higher animals the factors controlling intermediary metabolism may be considered at various levels, all of which are intimately related; for example, at the intracellular level there is the enzymatic regulation of metabolic pathways; the endocrine and nervous systems and the circulating levels of substrates and metabolites co-ordinate the cellular events in different tissues and organs; and these phenomena in turn are associated with the activities of the whole animal, such as feeding and exercise.

This thesis is concerned with one particular aspect of the regulation of carbohydrate metabolism - the role of sex hormones. The involvement of several other hormones, insulin, glucagon, growth hormone, corticosteroids, catecholamines, gastro-intestinal hormones and thyroid hormones has been extensively examined (Hales, 1967; Dickens, Randle & Whelan, 1968), but sex hormones have received little attention in this context.

The present volume evaluates the evidence for a relationship between sex hormones and carbohydrate metabolism, and reports a series of experiments which hopefully contribute towards a further understanding of this area.

Although the scope of this work is necessarily limited, it

should not be forgotten that factors which effect carbohydrate metabolism are likely to produce effects throughout intermediary metabolism. The latter effects are beyond the bounds of this thesis. Clinical aspects have been considered in some detail because the present work is particularly relevant to, and may be of ultimate benefit in, the practice of medicine.

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ABBREVIATIONS

Système International d'Unités (SI Units) have been employed in this thesis. The following abbreviations have also been used:

A.F.: aldehyde fuchsin

cyclic AMP: adenosine 3':5'-cyclic monophosphate

diabetes: diabetes mellitus; types of diabetes are defined after Oakley, Pyke & Taylor (1968)

EDTA: ethylenediaminetetraacetic acid

FSH: follicle stimulating hormone

GTT: glucose tolerance test

HCG: human chorionic gonadotrophin

H.E.: haematoxylin and eosin

HGH: human growth hormone

HMG: human menopausal gonadotrophin

HPL: human placental lactogen

ILA: insulin-like activity

ipGT_A: intraperitoneal glucose tolerance area

ipGTTs: intraperitoneal glucose tolerance tests

IRI: immunoreactive insulin

ivGTTs: intravenous glucose tolerance tests

LH: luteinizing hormone

methyl testosterone: 17 β -Hydroxy-17 α -methyl-4-androsten-3-one

oestradiol: 1,3,5(10)-Estratriene-3,17 β -diol

oestradiol benzoate: 1,3,5(10)-Estratriene-3,17 β -diol,3-benzoate

oGTTs: oral glucose tolerance tests

progesterone: 4-Pregnene-3,20-dione

saline: sodium chloride solution, 0.9% (w/v)

testosterone: 17 β -Hydroxy-4-androsten-3-one

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CHAPTER 1

INTRODUCTION

There is evidence in the literature to suggest that sex hormones - oestrogens, progestins, androgens and pituitary gonadotrophins may be associated with carbohydrate metabolism. Most of this evidence relates to the female and assumes a circumstantial nature, having been derived from observations that glucose homeostasis is modified during different reproductive states. Few studies have been specifically concerned with the topic and these have been conducted mainly in experimental animals. The rat has featured commonly in such studies and this animal model has been adopted in the present series of experiments.

The literature conveniently falls into clinical and non-clinical categories. Reports in the latter category pertain for the most part to the rat, but studies in other experimental animals have been included where they contribute further information. Data obtained in males and in females have been carefully distinguished because of the importance of sex differences. Considerable evidence relates to the condition of diabetes mellitus, a disorder characterised by abnormal carbohydrate metabolism and generally attributed to a net deficiency of insulin (Dickens et al., 1968; Malins, 1968; Oakley, Pyke & Taylor, 1968; Cerasi & Luft, 1970). Since diabetes is associated with alterations throughout intermediary metabolism, evidence from this source has been interpreted with particular caution.

Different reproductive states are known to correspond with alterations of sexual endocrine function, and carbohydrate metabolism has been evaluated in this context during the menstrual cycle and pregnancy in women, during pregnancy in the rat, and during pathological conditions of sexual activity in men and women. Circumstantial evidence obtained in this way has been compared with the effects of sex hormone preparations on carbohydrate metabolism in men, women and experimental animals. Abnormalities of reproductive competence in diabetes have been considered in relation to possible sexual endocrine lesions, and age-related changes of carbohydrate metabolism and sex differences of carbohydrate metabolism have been reviewed with respect to the prevailing sex hormone environment.

Numerous methods have been employed to assess carbohydrate metabolism, but no single method provides an adequate overall picture. This is substantially achieved only when a host of selected parameters from all levels of the organism are collated. Literature regarding sex hormones and carbohydrate metabolism does not provide a complete appreciation. It centres predominantly on the regulation of blood glucose homeostasis and the role of insulin, and few relevant studies have been performed at the level of the whole animal or on the cellular metabolism of glucose. The interaction of sex hormones and insulin in the regulation of blood glucose homeostasis forms a general theme throughout this thesis.

Menstrual Cycle

Fluctuations in the levels of circulating sex hormones during the menstrual cycle have been reviewed recently by Moghissi, Syner & Evans (1972).

Changes of glucose metabolism during the cycles of apparently normal healthy, non-diabetic women have not been established. Several studies of the fasting blood sugar level have failed to detect any consistent changes (see review by Spellacy, Carlson & Schade, 1967d; Reinke, Ansah & Voigt, 1972), and studies of glucose tolerance during the cycle are equally inconclusive (table 1). It is possible that the conflicting data reported in these studies are at least partially due to methodological differences.

Observations during the menstrual cycles of diabetic women, however, are in general agreement that the severity of the condition is increased at the time of menstruation when the sex hormones titers are low (Harrop & Mosenthal, 1918; Rosenbloom, 1921; Von Noorden & Isaak, 1927; Peperkorn, 1932; Cramer, 1942; Morton & McGavack, 1946; Hubble, 1954; Greene, 1958; Sandström, 1969; Leading article, 1970).

Pregnancy

Clinical

Pregnancy is attended by gross adjustments of the maternal endocrine system, including characteristic elevations of the circulating levels of oestrogens, progestins and adrenal cortical hormones. There is an early peak of chorionic gonadotrophin (HCG), a later and sustained rise of placental

lactogen (HPL), and a reduction of growth hormone (HGH) (Deanesly, 1966; Fuchs & Klopfer, 1971).

During the course of normal pregnancy insulin secretion appears to be increased, especially during the third trimester (Spellacy & Goetz, 1963; Bleicher, O'Sullivan & Freinkel, 1964; Kalkhoff, Schalch, Walker, Beck, Kipnis & Daughaday, 1964; Trayner, Welborn, Rubenstein and Russel-Fraser, 1967; Campbell, Pyke & Taylor, 1971). Fasting blood sugar levels tend to become reduced as pregnancy progresses, but glucose tolerance is generally unaffected or marginally impaired (Benjamin & Casper, 1966; Oakley & Peel, 1968; O'Sullivan, Snyder, Sporer, Dandrow & Charles, 1970; Campbell et al., 1971; Macdonald, Good, Schwartz & Stone, 1971a; Mestman, Anderson & Barton, 1971).

That pregnancy provides a diabetogenic stress has been appreciated for many years (Bergqvist, 1954; Kyle, 1963; Peel, 1972) and an apparent diabetic condition (gestational diabetes) may develop in women who are without any signs of abnormal carbohydrate metabolism in the non-gravid state. The diabetogenic effects of pregnancy are more often prevalent in potential and latent diabetics (Campbell et al., 1971). It appears that gestational diabetes is accompanied by a deficient insulin response to glucose, principally due to an inability of "acute" insulin release (Yen, Tsai & Vela, 1971).

The extra requirement for insulin during pregnancy is generally attributed to increased peripheral insulin resistance (Burt, 1956) which results from other endocrine influences, particularly the elevated levels of HPL and glucocorticoids (Beck & Wells, 1969; Kalkhoff, Richardson & Beck, 1969;

Fiorotelli, Gonazzani, Aubert, Gragnoli & Pupillo, 1970; Spellacy, 1971). At the same time the elevated levels of these hormones and also oestrogens and progesterone may contribute to a stimulation of the pancreatic beta cells (Kalkhoff, Jacobson & Lemper, 1970; Spellacy, 1971; Yen et al. 1971).

It has been observed that during the early puerperium there is a marked transient improvement of glucose tolerance and a reduction of circulating insulin (Love, Stevenson & Kinch, 1964; Burt, Leake & Rhyne, 1969; Spellacy & Buhi, 1969; Macdonald, Good, Schwartz & Stone, 1971b). These changes may be correlated with the endocrinological alterations at parturition. The factors attributed to insulin resistance and insulin stimulation during pregnancy are eliminated since there is a sharp reduction in the circulating levels of steroids and HPL, and the normal levels of growth hormone (an insulin antagonist) are only slowly regained (Spellacy & Buhi, 1969; Macdonald et al., 1971b).

In asymptomatic and clinical diabetic women the endocrine changes of pregnancy are essentially similar to those of normal women although the levels of oestrogens may be reduced and the levels of HPL may be raised (Cohen 1971; Lin, Lin, Erlenmeyer, Kline, Underwood, Billiar & Little, 1972; Ursell, Brudenell & Chard, 1973). Diabetic pregnancies are particularly hazardous for the mother, and are associated with a high incidence of obstetric and paediatric complications (Kyle, 1963; Oakley & Peel, 1968). The diabetic condition worsens during pregnancy as the insulin requirement increases and requires careful therapeutic control.

Rat

The endocrine aspects of pregnancy in the rat appear to be generally similar to those of human pregnancy (Deanesly, 1966; Heap, 1972). There is a rise in the circulating level of oestrogens (Yoshinaga, Hawkins & Stocker, 1969; Shaikh, 1971), progestins (Hashimoto, Henricks, Anderson & Melampy, 1968; Sutter-Dub, Leclercq, Felix, Jacquot & Sutter, 1973) and adrenal cortical hormones while the level of growth hormone declines (Schalch & Reichlin, 1966). There is also evidence for a rat placental lactogen (Gusdon, Leake, Van Dyke & Atkins, 1970).

Changes of glucose homeostasis in the pregnant rat are also similar to those of human pregnancy. There is a reduction of the normal blood sugar level (Herrera, Knopp & Freinkel, 1969; Malaisse, Malaisse-Lagae, Picard & Flament-Durand, 1969; Knopp, Ruder, Herrera & Freinkel, 1970; Costrini & Kalkhoff, 1971; Green & Taylor, 1972; Sutter-Dub et al., 1973) but glucose tolerance is apparently unaltered (Costrini & Kalkhoff, 1971). The circulating level of insulin becomes raised (Herrera et al., 1969; Costrini & Kalkhoff, 1971; Sutter-Dub et al., 1973) and this is associated with an increased peripheral insulin resistance (Knopp et al., 1970).

In vitro studies using pieces of pancreas (Malaisse et al., 1969) and isolated islets (Costrini & Kalkhoff, 1971; Green & Taylor, 1972) obtained from rats in late pregnancy have shown a markedly enhanced insulin secretory response to glucose stimulation compared with non-pregnant controls, and Green & Taylor (1972) have demonstrated that the threshold for a secretory response to glucose is lowered for islets from pregnant

rats. The total insulin content of the pancreas is also greater in the pregnant rat (Hoet & Lukens, 1954; Malaisse et al., 1969; Rishi, Golob, Becker & Shah, 1969) and the size, protein content and DNA content of the islets concomitantly increases (Costrini & Kalkhoff, 1971; Green & Taylor, 1972).

It has been suggested that the increased insulin secretory response of the islets of the pregnant rat may be at least partially due to the influence of circulating oestrogens and progestins (Costrini & Kalkhoff, 1971) and placental lactogen (Malaisse et al., 1969).

Experimental diabetes in rats is associated with complications of pregnancy analogous to those of human pregnancy. In both alloxan diabetes and partial-pancreatectomy diabetes there is an increased incidence of abortion and foetal mortality and a reduction of litter size: the diabetic condition worsens during pregnancy, and structural and metabolic changes of the pituitary, ovaries, uteri and placentae have also been observed (Lindan & Morgan, 1950; Angerwall, 1959; Kim, Runge, Wells & Lazarow, 1960; Foglia, Chieri & Ramas, 1970). An altered sexual hormonal pattern has been postulated to contribute towards the symptoms observed in the pregnant diabetic rat (Foglia et al., 1970).

Pathological conditions

Studies of carbohydrate metabolism in patients with pathological alterations of sexual endocrine function are very limited.

A preponderance of diabetes among hirsute women has been

reported, but it is unestablished whether increased androgenic activity is contributing to a diabetogenic effect (Malaisse, Lauvaux, Franckson & Bastenie, 1965). Logan Edwards (1973) has observed a flattened oral glucose tolerance curve in women with secondary amenorrhoea. The influence of the amenorrhoeic endocrine environment and in particular the hypothalamic-hypophyseal-ovarian lesion on this phenomenon is currently being investigated.

Effects of sex hormone preparations on glucose tolerance and circulating insulin levels

Non-diabetic women

Preparations of naturally occurring gonadal steroids do not appear to have a detrimental effect on glucose metabolism in women of child-bearing age: they may in fact improve glucose tolerance and elevate circulating insulin levels, depending on the nature of the preparation and the dosage and duration of treatment. However, small doses of oestradiol valerate or 17-hydroxyprogesterone caproate have not been found to produce any significant changes of glucose tolerance or insulin levels in post-menopausal women (table 2).

Evidence has been presented by Talaat, Habib, Higazy, Naby, Malek & Ibrahim (1965a) that oestradiol increases glucose utilization and enhances insulin sensitivity in pre-menopausal women. The same workers have also reported that glucose tolerance and insulin sensitivity in pre-menopausal women are unaffected by treatment with testosterone.

Diabetic women

There have been reports that preparations of naturally occurring oestrogens may improve the condition of pre-menopausal and post-menopausal diabetic women. These reports have described reductions of blood glucose and a decrease and stabilization of insulin requirement (Glen, 1938; Gessler, 1939; Spiegelman, 1940; Marcus & Glotzer, 1948). Talaat et al. (1965a) observed no significant alteration of glucose tolerance in a group of diabetic women treated with oestradiol dipropionate.

At present there is considerable controversy concerning the effects of synthetic oestrogens and progestins on glucose metabolism in women. It appears that the effects produced by these compounds are not always consistent with those produced by the natural hormones: this aspect is considered later (Appendix ii). The present section is concerned only with preparations of natural hormones.

Men

Healthy adult men receiving testosterone treatment do not exhibit any significant changes of glucose tolerance, although insulin sensitivity may be increased (Talaat, Habib & Habib, 1957).

Klinefelters syndrome (XXY male) is associated with an increased frequency of diabetes but it is uncertain whether this is related to the reduced androgenic titers of these patients. Following testosterone treatment non-diabetic Klinefelters showed no apparent changes of glucose tolerance or insulin levels although diabetic Klinefelters tended to show an improvement of glucose tolerance (Serrano Rios, Hawkins,

Escobar, Mato, Larrodera, Oya & Rodriguez-Minon, 1972). Two other reports have also suggested that testosterone might be beneficial to the condition of male diabetics (Pellegrini, 1941; Kinsell, Morgan & Michaels, 1949). Kalkhoff, Jacobson & Lemper (1970) have reported that progesterone increases insulin levels but does not alter blood glucose in normal men.

Effects of sex hormones on aspects of carbohydrate metabolism in sub-human species

Glucose tolerance and circulating insulin levels

Early studies of the effects of ovarian steroids on the blood sugar level of non-diabetic female animals used comparatively impure ovarian extracts and a variety of different treatment regimes (see reviews by Beric & Karanfilski, 1957; Benjamin & Casper, 1966). Oestrogenic preparations were reported to exert a hyperglycaemic effect by some authors and a hypoglycaemic effect by others, but progestogenic preparations consistently produced a hypoglycaemic effect.

In more recent studies it has been observed that oestradiol lowers blood sugar levels in rats (Basabe, Chieri & Foglia, 1969) and in rabbits (Beric & Karanfilski, 1957; Talaat, Habib & Malek, 1960) and an improvement of glucose tolerance has been reported in oestradiol treated rabbits and chickens (Planas, Martin-Mateo & Laguma, 1967). Talaat et al. (1960) have indicated that the effects of oestradiol on blood sugar levels in rabbits are dose dependent. Beck (1969a) was unable to identify a significant alteration of glucose tolerance or plasma insulin levels in Rhesus monkeys treated with oestradiol or oestriol, but Basabe et al. (1969) observed a

hyperinsulinaemic influence of oestradiol in rats.

Treatment of Rhesus monkeys with progesterone enhanced plasma insulin responses to intravenous glucose without altering glucose tolerance (Beck, 1969b). These effects of progesterone may be modified by the presence of oestriol, oestradiol and human placental lactogen (Beck & Hoff, 1971). Basabe et al. (1969) did not observe any significant influence of progesterone on blood sugar levels and plasma insulin levels in rats.

It has been reported that testosterone treatment does not alter blood sugar levels in dogs and rats (Sirek & Best, 1953; Basabe et al., 1969) and does not produce any apparent effect on glucose tolerance in rabbits (Planas et al., 1967). After six months of testosterone treatment, Basabe et al. (1969) noted that plasma insulin levels tended to become raised in their group of rats.

Talaat and co-workers (1958; 1961; 1965) have studied the effects of two gonadal steroids on aspects of glucose metabolism in non-diabetic male rabbits. They observed that testosterone raises the basal blood sugar level and impairs glucose tolerance, and that oestradiol also tends to produce a hyperglycaemic effect.

Feeding, body weight and activity

It is well established that there are sex differences in body weight, food intake and locomotor activity in rats. Males weigh and eat more and exercise less than do females (see review by Wade, 1972).

Oestradiol appears to be the principal ovarian steroid regulating body weight in the female rat: it depresses food intake and promotes locomotor activity. Progesterone tends to attenuate the influence of oestradiol and treatment with large doses of progesterone increases eating and body weight and depresses running activity.

In male rats testosterone induces weight gain, eating and running activity.

Glycogen deposition

Several studies have shown that oestrogen preparations cause an accumulation of hepatic and uterine glycogen in female rats without causing any significant alterations in skeletal muscle glycogen (Song & Kappas, 1968; Villar-Palasi, 1968; Paul, 1971; Matute & Kalkhoff, 1973). Paul & Duttagupta (1973) reported that progesterone attenuates these effects of oestrogens and reduces uterine glycogen but not hepatic glycogen when administered alone. However, Matute & Kalkhoff (1973) claim that progesterone enhances hepatic glycogen content. Leonard (1952) observed an increased glycogen content of skeletal muscle in testosterone treated female rats.

Testosterone has been shown to promote the deposition of glycogen into skeletal muscle of male rats (Leonard, 1952) and into muscle and liver in male rabbits (Talaat, Habib & Hanna, 1958).

Glucose utilization and insulin sensitivity

Several studies have demonstrated that oestrogens increase glucose uptake and utilization in rat uterus (see review by

Warren, 1971).

Gilmour & McKerns (1966) have observed that oestradiol is synergistic with insulin in promoting glucose uptake and conversion to lipid by the adipose tissue of the female rat. Progesterone, dehydroepiandrosterone and testosterone, however, were without any significant effect. Adipose tissue of the male rat was not responsive to the presence of oestradiol. This study is consistent with the report of Talaat et al. (1965a) that oestradiol improves glucose utilization and insulin sensitivity in women.

In the female Rhesus monkey, Beck (1969) has noted that progesterone impairs insulin sensitivity, which is consistent with the observations in monkeys and in humans that progesterone may raise plasma insulin levels without altering glucose tolerance.

Morphology and insulin content of the pancreatic islets

Female rats treated with oestrogens or progesterone exhibit hyperplasia and hypertrophy of the pancreatic islets. This is associated with an increased insulin content and a decrease in the ratio of alpha cells to beta cells (Bottiglioni & Flamigni, 1963; Haist, 1965). A similar effect has been observed after HCG treatment (Bottiglioni & Flamigni, 1963), but FSH exerts only a mild effect and LH is apparently inactive (Melloni & Pecchiri, 1957).

No significant changes of islet morphology or insulin content have been noted in male rats with testosterone (Bottiglioni & Flamigni, 1963; Haist, 1965).

Experimental diabetes

The effects of sex hormones on the incidence and severity of experimental diabetes have been studied using partially pancreatectomized rats. These studies have been substantially reviewed by Houssay (1951) and Rodriguez (1965).

Foglia, Schuster & Rodriguez (1947) reported that the incidence of diabetes was higher in males than in females and a possible influence of gonadal steroids was indicated by the observation that orchidectomy reduced the incidence in males while ovariectomy increased the incidence in females.

In female rats that have been ovariectomized and partially pancreatectomized, treatment with oestrogens appears to provide a protection against the diabetes (Lewis, Foglia & Rodriguez, 1950) although this effect may be preceded by an initial transient hyperglycaemia (Houssay, Foglia & Rodriguez, 1954). Since the protective influence of oestrogens is effective in both adrenalectomized and hypophysectomized animals, it has been suggested that the adrenal and pituitary glands are not implicit in the protective mechanism (Foglia, Penhos & Cardeza, 1954; Houssay et al., 1954). The local hyperplasia and hypertrophy of the pancreatic islets observed when pellets of oestradiol were embedded within cat pancreata (Cardeza & Rodriguez, 1949) indicated a possible direct action of oestrogens on the pancreatic islets, but this work has been criticised by Haist (1965) on the evidence that non-specific injury of the pancreas may induce the same effect.

In contrast to the effect of oestrogens, moderate doses of

progesterone were without any apparent effect, while testosterone markedly increased both the incidence and the severity of experimental diabetes in ovariectomized female rats (Lewis et al., 1950).

The diabetes of orchidectomized and pancreatectomized male rats was also aggravated by testosterone and ameliorated by oestrogens (Lewis et al., 1950).

Effects of diabetes on reproductive function

Sexual disturbances are known to occur more frequently in diabetic men than in non-diabetic men. These disturbances include impotence, atrophy of the genitalia and a reduced spermatogenesis (Rubin & Babbott, 1958; Klebanow & Macleod, 1960; Schoffling, 1965; Irisawa, Shirai, Matsuschita, Kagayama & Ichijo, 1966). The plasma testosterone level in diabetic men, however, does not appear to be altered (Kent, 1966; Antonini & Petruzzi, 1970; Faerman, Vilar, Rivarola, Rosner, Jadzinsky, Fox, Perez Lloret, Bernstein-Hahn & Saraceni, 1972).

Impaired sexual function in diabetic women has also been observed: Kolodny (1971) has noted a high incidence of inability to reach sexual orgasm in these patients, and reduced fertility and complicated pregnancy in diabetic women have been mentioned in a previous section of this chapter.

There is also evidence that puberty is delayed in diabetic children of both sexes (Pond & Oakley, 1968).

Reports have appeared of impaired reproductive function in experimental diabetic rats. Atrophy of the testes and secondary sex glands, and a reduced insemination capacity have been

observed in both alloxan treated and partially pancreatectomized diabetic adult male rats (Foglia, Rosner, Ramos & Lema, 1969), and retarded sexual development has been noted in alloxan diabetic immature male rats (Hunt & Bailey, 1961).

In the diabetic female rat delayed sexual maturity, irregular oestrous cycles, degenerative changes of the ovaries and genital tracts, and reduced fertility have been reported (Davis, Fugo & Lawrence, 1947; Sonlairac, Desclaux & Katz, 1948; Lawrence & Contopoulos, 1960; Chieri, Pivetta & Foglia, 1969).

Although the mechanisms underlying sexual dysfunction in diabetes are unestablished it has been indicated that reduced secretion of pituitary gonadotrophins (Howland & Zebrowski, 1972) and an impaired response of the gonads to these hormones (Lin, Lin & Johnson, 1972) may occur in the diabetic rat. Irisawa et al., (1966) have also indicated a reduced secretion of pituitary gonadotrophins in human diabetics.

Ageing

Clinical

It is well established in man that glucose tolerance progressively deteriorates with advancing age (see review by Huerga & Sherrick, 1971) but concomitant changes in the glucose-insulin interrelationship are not, however, clear.

Some workers claim that the insulin response to glucose is the same in the elderly as in the young (Welborn, Rubenstein, Haslam & Fraser, 1966; Boyns, Crossley, Abrams, Jarrett & Keen, 1969; Johansen, 1972): other workers report a reduction of the insulin secretory response to glucose with age, suggesting a

defective mechanism of insulin synthesis and/or release (Crockford, Harbeck & Williams, 1966; Tobin, Andres, Sherwin, Liljenquist & Weissman, 1970): a third school maintain that the insulin response to glucose is increased with age, and attribute this to insulin resistance and/or insulin antagonism (Chlouverakis, Jarrett & Keen, 1967; O'Sullivan, Mahan, Freedlender & Williams, 1971; Schreuder, 1972). Woldow, Shapiro, Cohen & Kollman (1972) have observed normal, diminished and elevated insulin levels in elderly subjects with non-diabetic glucose curves.

Several groups have presented evidence that exogenous insulin is equally effective at lowering blood glucose in healthy individuals of all ages (Silverstone, Brandforbrenen, Shock & Yiengst, 1957; Calloway & Kujak, 1971; Tobin et al., 1970), but Marigo (1968) has reported a decreased sensitivity of the tissues to insulin in the aged. On the basis of this and histological studies of the pancreatic islets, Marigo (1968) has postulated that the changes of carbohydrate metabolism during age involve a phase of hyperinsulinism aimed at balancing a decreased insulin sensitivity of the tissues followed by a phase of hypoinsulinism owing to beta cell exhaustion. The hypothesis offers the possibility that the conflicting results of other workers may reflect the predominant phase within their respective groups of subjects.

Hayner, Kjelsberg, Epstein & Francis (1965) and Zeytinoglu, Gherondache & Pincus (1969) have noted that several features of normal glucose tolerance in the aged are reminiscent of mild maturity-onset diabetes in younger persons, and studies of the aetiology of the one might be applicable to the other.

It is tempting to speculate that the deterioration of glucose tolerance with age may be associated with concurrent alterations of other endocrine factors (see review by McGavack, 1967) and Zeytinoglu & Gherondache (1970) have already suggested that the hormone changes in post-menopausal women play a role in the impairment of glucose tolerance.

Available evidence does not indicate that glucose metabolism in non-diabetic women is especially influenced by the menopause (Butterfield, 1964; Hayner et al., 1965; Boyns et al., 1969), although a specific study of this aspect has not been reported.

It is possible that endocrinological alterations during infancy and adolescence, particularly the appearance of sex hormones (Heald & Hung, 1970), may be associated with changes of glucose metabolism, but surveys of individuals in this age range have not been concerned with this correlation (Rosenbloom & Allen, 1973; Cole, 1973).

Age-related aspects of the incidence of diabetes have been reported on several occasions. An arbitrary evaluation of previously published data (Fitzgerald, Malins, O'Sullivan & Wall, 1967; Pond & Oakley, 1968; Fajans, Floyd, Conn & Pek, 1970) does not indicate that the incidence of diabetes during infancy and adolescence is especially related to the endocrine events of puberty, but such an evaluation is complicated by the possibility that puberty is delayed in diabetic children (Pond & Oakley, 1968).

During adult life the incidence of diabetes increases with

advancing age and rises steeply at about the age of 45 and older. Since the high prevalence of diabetes in this age group is approximately the same for both sexes, a direct association with the menopause in women is not indicated (Fitzgerald et al., 1961). Similarly, a statistical evaluation of the mortality from diabetes during "midlife" does not implicate a special association with the menopause (Metropolitan Life Insurance Company, 1970).

The post-menopausal incidence of diabetes in women correlates positively with parity; that is, diabetes occurs more frequently in women who have had a greater number of pregnancies (Pyke, 1956; Fitzgerald et al., 1967) but the reason for this is unknown. Blood sugar values among the general population do not appear to be correlated with parity (United States Department of Health, Education and Welfare, 1966).

Thus, considerable evidence indicates a deterioration of the glucose homeostatic mechanism with age, but a possible relationship with sexual endocrine activity has not been specifically investigated and cannot be adequately evaluated from available data.

Rat

Consistent with the observations in man, it has been demonstrated that glucose tolerance deteriorates with advancing age in the rat (Hajdu, Herr & Rona, 1968; Klimas, 1968; Frolkis, Bogatskaya, Bogush & Shevchuk, 1971; Gommers, 1971; Gommers & De Gasparo, 1972). According to some workers (Klimas, 1968; Frolkis et al., 1971; Gommers, 1971; Gommers & De Gasparo, 1972; Hebold & Blenal, 1973) the fasting blood glucose level in adult rats does not alter significantly with age, but Hajdu et al.,

(1968) and Berdanier, Marshall & Moser (1971) have reported a definite increase.

Berdanier et al., (1971) also report an increase in the fasting IRI level with age (50 to 300 days). Gommers & De Gasparo (1972) have measured IRI values during ivGTTs at 3, 12 and 24 months of age. These workers observed a significant rise in the fasting IRI value at 12 months but not at 24 months. They also found that the insulin response to glucose was raised at 12 months and at 24 months, but to a much greater extent at the former time. These data may be considered in the context of Marigo's (1968) two-phase hypothesis of hyperinsulinism followed by hypoinsulinism.

Frolkis et al. (1971) produced evidence in rats up to 30 months of age that both the beta cell response to glucose and the sensitivity of the tissues to insulin diminishes with age. However, they also reported that in older animals there is an increase in the levels of insulin in the blood, associated with a reduced liver insulinase activity and a reduced activity of the circulating insulin.

The number and volume of the islets, and the insulin content of the pancreas of the rat have been observed to increase with age (up to 480 days) (Haist & Pugh, 1948; Hellman, 1959a,b,c). Hajdu & Rona (1967) have noted insular fibrosis in one year old male (but not female) rats. Similar fibroses have been reported in the islets of maturity-onset diabetics and non-diabetic aged humans (Ehrlich & Ratner, 1967), and in the islets of genetically obese-hyperglycaemic mice (gene type Obob) (Atkins, 1972).

Sex differences

It has been reported that the blood glucose levels during oGTTs are generally higher in women than in men (Boyns et al., 1969; Zeytinoglu et al., 1969). Other studies, however, have failed to detect a sex difference (Hayner et al., 1965; Welborn, Rubenstein, Haslam & Fraser, 1966; Hales, Greenwood, Mitchell & Strauss, 1968; Marine, Vinik, Edelstein & Jackson, 1969) and Soeldner, Cannon & Gleason (1971) have reported that the blood glucose levels during both oral and intravenous tests are higher in males. Higher serum or plasma insulin levels during glucose tolerance tests in women have been noted on several occasions (Boyns et al., 1969; Zeytinoglu et al., 1969; Welborn, Stenhouse, Curnow & Johnstone, 1970), but Hales et al. (1968) and Soeldner et al. (1971) were unable to find any consistent sex differences.

A sex difference in the incidence of diabetes is also uncertain since several conflicting reports have appeared (Fitzgerald et al., 1967; Pond & Oakley, 1968; Pyke 1968), and there is evidence that the male to female ratio of diabetes is changing (Malins, Fitzgerald & Wall, 1965; Nicholson, 1971). It is possible that social, environmental and diagnostic factors are masking any inherent sex differences.

Evidence concerning a sex difference in diabetes mortality is equally confused, and again there is an indication that the male to female ratio is changing (Malins et al., 1965; Pyke, 1968; Metropolitan Life Insurance Company, 1972). Statistical analyses of diabetes mortality are particularly difficult to interpret because diabetes is not always recognised as a cause

of death (Malins et al., 1965) and diabetic mortality per se is now frequently obviated by therapeutic means.

There have been only a limited number of observations relating to sex differences of carbohydrate metabolism in the rat. Devel, Gulick, Grunewald & Cutler (1934) noted that the fasting liver glycogen level was lower in female rats than in male rats, but there was no apparent sex difference in non-fasting animals. Hebold & Bleuel (1973) found that the fasting blood glucose level was higher in female rats than in male rats, and that the sulphonylurea HB 419 produced a greater, more rapid and more prolonged hypoglycaemia in the former sex. Following partial pancreatectomy male rats develop diabetes much more frequently than female rats (Foglia, Shuster & Rodriguez, 1947; Beach, Cullimore & Bradshaw, 1957) but a sex difference opposite to this occurs in alloxan treated rats (Beach, Bradshaw & Blatherwick, 1951).

According to Tejning (1947) the average size of the pancreatic islets is greater in female rats than in male rats, but Haist (1965) was unable to verify this work. Hajdu & Rona (1967) noted that the incidence of spontaneous insular fibrosis in a colony of ageing Sprague-Dawley rats was almost exclusively confined to the males; and they later attributed this to a protective action of oestrogens in the female (Hajdu & Rona, 1971). Thus, although aspects of carbohydrate metabolism have been reported to vary between male and female rats, a consistent pattern of sex differences has not yet emerged.

Comment

The evidence presented in this review has provided a basis for proposing the general hypothesis that 'sex hormones participate in the physiological regulation of carbohydrate metabolism'.

In the female it appears that preparations of sex steroids do not produce a detrimental effect on the overall metabolism of carbohydrates, and they may induce changes of a beneficial nature, such as improved glucose tolerance. The activities of these preparations seem to be related to the precise structural properties of the molecule, the administration regime and the physio-pathological condition of the recipient. Although it is difficult to assess the contributions of various sex hormones towards changes of carbohydrate metabolism during different reproductive states, available evidence largely indicates that the endogenously secreted hormones tend to produce similar effects to the exogenously administered compounds.

Sex steroids undoubtedly influence glycogen deposition in the male, but isolated reports that they modify other aspects of carbohydrate metabolism remain unsubstantiated.

Several studies have implied an alteration of sexual endocrine function in diabetic individuals. This evidence is considered to support the hypothesis.

The age-related changes of carbohydrate metabolism are only partially understood, and sex differences of carbohydrate metabolism have not yet been conclusively established. It is possible that the prevailing sex hormone environment bears an

important influence on these phenomena.

Experiments reported in this thesis are an examination of certain aspects of the hypothesis. The effects of individual sex hormones on selected parameters of carbohydrate metabolism have been investigated in male and female rats. These effects have been compared with changes that occur during different reproductive states, and a preliminary study has been conducted on the mechanisms involved.

TABLE 1. GLUCOSE TOLERANCE DURING THE MENSTRUAL CYCLE

AUTHOR(S)	Year	Type of GTT	Days of Menstrual Cycle on which tests were performed					CONCLUSIONS
			0	14	28			
Heilig	1924	0	arbitrary times					Improved GT around ovulation
Okey & Robb	1925	0	arbitrary times					Improved GT at menses; impaired GT immediately before & immediately after the menses
Garufi & Ruggeri	1933	0	arbitrary times					Improved GT around ovulation impaired GT pre-menstrual
Asinelli & Casassa	1937	0	arbitrary times					impaired GT pre-menstrual
Frerichs et al.	1966	iv	2	8	20	impaired GT on day 8		
Pyorala et al.	1967	iv	5 - 6	12 - 14	19 - 24	No changes of GT		
Spellacy et al.*	1967d	iv	4 - 6		24-26	No changes of GT No changes of plasma insulin levels		
Jarrett & Graver	1968	0	arbitrary times					GT improved at menses GT impaired at & after ovulation
Taylor & Kass	1968	0		15	28	No changes of GT		
Larsson-Cohn* et al.	1969	iv	Follicular		luteal	No changes of GT No changes of plasma insulin levels		
Macdonald & & Crossley	1970	0	1 - 6	7 - 12	13 - 18	19 - 24	25+	GT improved at ovulation & steadily deteriorates until the time of ovulation of next cycle

* measured plasma insulin levels during GTT

TABLE 2. EFFECT OF PREPARATIONS OF NATURALLY OCCURRING OESTROGENS AND
PROGESTINS ON GLUCOSE METABOLISM IN NON-DIABETIC WOMEN

Author(s)	Hormone Treatment	Type of GTT	Glucose tolerance	Circulating insulin
	<u>Pre-menopausal, apparently normal</u>			
Beric et al. 1957	oestradiol dipropionate 5 mg i.m. 2 hours before	0	improved	-
Talaat et al. 1965a	oestradiol dipropionate 25 mg i.m. 12 hours before	iv	unchanged	-
"	oestradiol dipropionate 1 or 5 mg i.m. alternate days, 17 to 20 days	iv	improved	-
Schreibman 1968	17-hydroxyprogesterone caproate 250mg i.m. 12 hours before	0	unchanged	elevated
Goberna et al. 1972	oestradiol .33mg/kg i.m. 5 days before	iv	unchanged	elevated
"	17-hydroxyprogesterone 5 mg/kg i.m. 5 days before	iv	unchanged	elevated
	<u>Pre-menopausal, endometrial carcinoma</u>			
Benjamin & Casper 1966	17-hydroxyprogesterone caproate 250 mg i.m. 5 days before	0	improved	-

Table 2 (continued)

	<u>Pre-menopausal, hysterectomized, ovaries intact</u>		
Kalkhoff et al.1970	progesterone 300 - 400 mg/day i.m. for 6 days	0	unchanged elevated
	<u>Post-menopausal, apparently normal</u>		
Schreibeman, 1968	17-hydroxyprogesterone caproate 250 mg i.m. 5 days before	0	unchanged unchanged
Pyorala et al.1971	oestradiol valerate 2 mg/day orally for 20 days, with a pause of 8 days, for 1, 3-4 and 8-12 months	0	unchanged unchanged

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

The following sections concern general materials and methods employed in the present study. In certain experiments these methods have been modified and other procedures have been used. Full details of these experiments are given in the relevant chapters.

Animals

Albino rats (Rattus norvegicus albinus) of the Wistar strain were used throughout. The animals were purchased from Fisons Pharmaceuticals Ltd., Loughborough, and accommodated in the University of Aston animal house for at least 3 weeks prior to experimentation. Groups of 4 animals were housed per cage (48 x 30 x 15 cm), and the bedding of wood shavings and straw was changed daily. The room was air-conditioned at $25 \pm 2^{\circ}\text{C}$ with a regular lighting schedule of 10 hours light (09.00 to 19.00 hours) and 14 hours darkness. Tap water and a standard pellet diet (diet 41B, L.A. Pilsbury, Birmingham) were supplied ad libitum.

Vaginal Smears

The different stages of the oestrous cycle of the adult female rat were determined by the vaginal smear technique (Zarrow, Yochim & McCarthy, 1964). Young adult females have a regular 4-day cycle, each day of which is associated with a characteristic vaginal smear. In ageing females the cycle tends to become irregular and the stage of dioestrus may be

extended over several days. The stages of the cycle were defined as follows: pro-oestrus, the day on which nucleated epithelial cells with a few cornified epithelial cells were present in the smear; oestrus, the day on which only cornified epithelial cells were present in the smear; metoestrus, the day on which large numbers of leukocytes and fragmented cornified epithelial cells were present in the smear; and dioestrus, the day on which only a few leukocytes and fragmented cornified epithelial cells were present in the smear.

Surgical procedures

Surgical procedures for recovery experiments were carried out with the maximum amount of asepsis possible. Operations were performed in an operating theatre using a temperature controlled (27°C) stainless steel operating table and a standard Boyles apparatus for dispensing volatile anaesthetics (The British Oxygen Co. Ltd., London) fitted with a miniature face mask. Anaesthesia was induced by inhalation of a gaseous mixture of 80% nitrous oxide and 20% oxygen containing 3.5% (v/v) halothane (Fluothane, ICI Ltd., Macclesfield). Anaesthesia was maintained with 1 to 1.5% (v/v) halothane in the same gaseous mixture. Instruments and sutures were sterilized in a mixture of 5% Hibitane (ICI Ltd.) and 70% methyl alcohol 2:5 v/v, and rinsed in a solution of sterilized saline (Steriflex, Vestric Ltd., Brierley Hill) before contact with the animal tissues. The operation site was washed with Hibitane-methyl alcohol and sterilized saline before and after surgery. To minimize post-operative infection animals were housed for 2 to 3 days post-operatively in clean cages bedded with absorbent paper, and wounds were treated with sulphathiozole and penicillin (5,000 iu/g) (Richard Daniel & Son Ltd., Derby).

Ovariectomy

Bilateral ovariectomy of young adult female rats was performed after D'Amour, Blood & Beldon (1969). The animal was anaesthetized and the operation sites were shaved and washed. Each ovary was removed by the following procedure. The abdomen was opened by a length-wise incision of about 1 cm in the mid-lateral region. The ovary embedded in the parametrial fat was located towards the dorsal muscle mass and was carefully exteriorized. The fallopian tube and associated fat and blood vessels were doubly ligated (size 0000 polyamide suture, Armour Pharmaceutical Company Ltd., Eastbourne), and the tissue was transected between the two ligatures. The ovary was removed, together with some parametrial fat and the upper portion of the fallopian tube, and the remaining tissue was returned into the abdomen. The muscle wall was closed with 2 or 3 sutures (size 000) using a curved triangular needle (size 14) and the skin was closed in a similar way.

Orchidectomy

Bilateral orchidectomy (castration) of young adult male rats was performed after D'Amour et al. (1969). The scrotum of the anaesthetized animal was washed and opened by a mid-line incision of about 1 cm. If the testes were retracted into the abdominal cavity they were forced back into the scrotum by slight pressure over the pelvis. Each testis was extruded through a small opening cut in the surrounding tunica, and the spermatic cord and associated blood vessels were doubly ligated (size 0000 suture) between the cauda epididymis and the epididymal fat pad. The tissue was cut between the two ligatures

and the testis and epididymis were removed. The scrotum was then closed with 2 or 3 sutures (size 0000).

Blood Sampling

Several methods have been described for the collection of repeated blood samples from conscious rats with the minimum of trauma. These methods were evaluated with particular reference to the requirements of intraperitoneal glucose tolerance tests. The methods were: puncture of the orbital sinus (Stone, 1954); permanent cannulation of the carotid artery (Popovic & Popovic, 1960; Jacobs & Adriaensseus, 1970); puncture of the midventral caudal artery (Hurwitz, 1971); and various modifications of the tail-tip amputation method (Grice, 1964; Porter, 1967; Wright, 1971; Atkins & Thornburn, 1971).

The tail-tip amputation procedure of Grice (1964) was found to be the most suitable, and was adapted as described below. The rat was introduced into a cylindrical plastic restrainer with the tail protruding (fig.1). The restrainer was adjusted to the length of the animal by the extent of insertion of the rubber bung. Animals were brought into contact with the restrainer on several occasions before an experiment so that they became accustomed to the apparatus and usually entered it voluntarily. The extreme tip of the tail was cut off with a sharp scalpel blade and blood was "milked" drop-wise from the wound by applying gentle pressure with a downwards motion of the hand over the tail. If necessary bleeding was stemmed by the application of moderate pressure to the tip of the tail. Although blood clots quickly at the cut tail-tip the wound may be re-opened for further sampling up to an hour later by

repeating the "milking" action. The procedure did not appear to stress the animal.

During intraperitoneal glucose tolerance tests 50 μ l samples of blood were collected in approximately one minute from the time the rat was removed from the cage until the time it was returned. Larger samples of blood (approximately 1 ml) required for triplicate determinations of plasma insulin levels were collected within three to four minutes.

Selection of parameters of carbohydrate metabolism

In vivo studies

Methods designed to evaluate the efficiency of blood glucose homeostatic mechanisms mostly depend upon the ability to re-establish a normal blood glucose level following a particular hyper- or hypo-glycaemic stimulus. An indication of the importance of insulin may be obtained by the determination of concomitant changes in the circulating level of this hormone (Ferriman & Gilliland, 1968; Malins, 1968; Roeckel, 1971). The most extensively explored method is the glucose tolerance test, which provides a profile of glycaemic alterations in response to a specific glucose challenge. Several different types of glucose tolerance test have been described, differing for the most part in the dose and route of administration of the glucose, and the site and frequency of blood sampling. The tests have been interpreted principally in connection with the diagnosis of diabetes, to estimate the rate of glucose entry into and/or removal from the blood (Chandalia & Boshell, 1970; Kobberling & Creutzfeldt, 1970; Billewicz, Anderson & Lind, 1973; Duffy, Phillips & Pellegrin, 1973; Olefsky, Farquhar & Reaven, 1973;

O'Sullivan & Mahan, 1973; Yudaken & Bloomberg, 1973). The relationship between blood glucose and insulin levels during the tests has been examined on many occasions and a variety of analytical procedures have been proposed. However the implications of data obtained in this way remain uncertain (Corte, Romano, Voeghelin & Serio, 1970; Atkins, 1971).

Glucose tolerance tests are affected by the physio-pathological condition of the individual: many metabolic variables, diseases, and specific and non-specific forms of stress have been shown to distort the results (Report of the committee on statistics of the American Diabetes Associations, 1969; Duffy et al., 1973). Since it is not possible to take all of these factors into account, the conditions of the tests must be rigidly standardized.

In the present series of experiments two kinds of glucose tolerance tests have been employed to investigate glucose homeostasis and the interaction of insulin.

Intraperitoneal glucose tolerance tests

Intraperitoneal glucose tolerance tests (ipGTTs) were performed at 15.00 hours on animals previously deprived of food for 6 hours. Animals were weighed at least an hour before the test. Blood samples (50 μ l) were collected from the cut tip of the tail immediately before, and exactly 15, 30 and 60 minutes after an intraperitoneal injection of glucose (2 g/kg body weight in a 40% (w/v) solution at 37°C). Blood sugar concentrations were determined as described in a later section of this chapter.

Food and water were withdrawn during the test and the

animals were housed individually in small cages (27 x 20 x 10 cm) bedded with absorbent paper. Precautions were taken to prohibit exogenous distractions of a potentially stressful nature, such as loud noises and unfamiliar smells, and the animals always remained quiescent. Environmental factors were the same for all tests.

To facilitate the comparison of glucose tolerance tests the results have been expressed as a function of the area beneath the glucose curve (after Jarrett & Graver, 1968) which is proportional to $\frac{a + d}{2} + b + c$, where a, b, c and d were the blood sugar values at 0, 15, 30 and 60 minutes respectively. The area thus measured is bounded by straight lines joining the 4 points, the ordinates and the abscissa, and has been termed the glucose tolerance area ($ipGT_A$). The function derived is effectively the sum of the blood sugar values at 15 and 30 minutes and the average of the values at zero and 60 minutes. Thus the relative contribution of the lower blood sugar values (zero and 60 minutes) has been reduced to allow emphasis of the 15 and 30 minute values, which are the principle variants in the tests (fig.2).

Studies of intraperitoneal glucose tolerance were supplemented with determinations of plasma insulin levels in identically treated animals. Blood samples (approximately 1 ml) were collected at 15.00 hours from animals previously deprived of food for 6 hours and weighed at least an hour before sampling. The blood was collected into polystyrene tubes that had been pre-washed with a solution of saline-heparin (80 U/ml). Plasma was separated by centrifugation and triplicate aliquots of

100 μ l were transferred to radioimmunoassay tubes and stored at -20°C until assayed.

The procedures were carried out at 15.00 hours on animals previously deprived of food for 6 hours to minimize the disturbance of the normal feeding routine. This was essential for the oestrous cycle study, since changes in food availability are accompanied by changes in cyclic activity (Cooper & Haynes, 1969). The same regime was adopted in subsequent experiments to enable a direct comparison of data. Laboratory rats feed and exercise during the hours of darkness and rest during the hours of light (Siegel, 1961). Thus the withdrawal of food between 09.00 and 15.00 hours does not interrupt scheduled feeding. The circadian pattern of circulating glucose and insulin levels has been studied in laboratory mice (Gagliardino & Hernandez, 1971; Jolin & Montes, 1973). By extrapolation of these data it appears that at 15.00 hours blood glucose and plasma insulin levels are approximately mid way between their normal upper and lower limits.

Intravenous glucose tolerance tests

Intravenous glucose tolerance tests (ivGTTs) were performed in which the changes of plasma insulin were measured in carotid arterial blood. Using this procedure a more valuable estimate of the insulin response to glucose loading may be obtained than by measuring the insulin level in peripheral venous blood. It is appreciated that the values derived in this way are a composite mixture of the insulin content of the peripheral venous return and the newly secreted pancreatic insulin that has not been metabolized by the liver (Kanazana,

Kuzuya, Ide & Kasaka, 1966; Rubenstein, Pottenger, Mako, Getz & Steiner, 1972). Similarly, the concentration of glucose in the arterial blood will comprise the component of peripheral venous return and the supplement provided by the liver (and intestine) (Exton, 1972).

Animals were fasted overnight, weighed and anaesthetized with sodium pentobarbitone (Nembutal, Abbott Laboratories Ltd., Queenborough, Kent), 45 mg/kg body weight administered intraperitoneally. The left carotid artery was cannulated for the withdrawal of blood samples and the right jugular vein was cannulated for the administration of test solutions after D'Amour et al. (1969). Each cannula consisted of a one-inch length of polythene tube (PP25, Portex Ltd., Hythe, Kent) attached by a 25G needle to a graduated 1 ml syringe. The cannulae were filled with a solution of saline-heparin (80 U/ml) which was prevented from mixing with the blood by a small air bubble (fig.3).

To withdraw a blood sample from the carotid cannula the syringe was removed, allowing the cannula to fill with blood at the expense of the saline-heparin solution. A clean 1 ml graduated syringe was attached and exactly 0.5 ml of blood was withdrawn. The original syringe was then re-attached and the blood, separated from the saline-heparin by an air bubble, was forced back down the cannula.

To administer a test solution via the jugular cannula, the saline-heparin solution was slowly aspirated from the cannula so that it was replaced with blood. A syringe containing the test solution was then attached and the test solution was

injected cautiously, taking care to avoid overloading of the cardiovascular system. The original syringe was re-attached and the saline-heparin solution, preceded by an air bubble, was returned to its original position in the cannula.

In all experiments the intravenous glucose challenge was 0.8 mg/g body weight in a 40% (w/v) solution, administered by an injection lasting approximately 30 seconds. Blood samples were obtained immediately before and at exactly 10, 20 and 30 minutes following the beginning of the glucose injection, and were analysed for blood sugar and plasma insulin as described below.

Unless otherwise stated the results obtained with different experimental groups were evaluated by comparing the summated values of blood sugar or plasma insulin at 10, 20 and 30 minutes.

In vitro studies

To substantiate data obtained from in vivo experiments, insulin secretion has been examined in vitro.

Methods for studying insulin secretion in vitro

In vitro preparations have contributed greatly to our appreciation of the factors that regulate insulin secretion and the cellular mechanisms that comprise the secretory process. The area has developed considerably during the last decade following the advent of insulin radioimmunoassay techniques, and although a variety of different preparations have been studied the results obtained are in general agreement.

Five basic in vitro methods may be distinguished, and the salient features of each of these methods together with their advantages and disadvantages are discussed in the following review. A more detailed consideration of two of these methods, the incubation of pancreas pieces and perfusion of the isolated pancreas is presented later since these methods have been employed in the present studies.

Incubation of pancreas pieces

Although Bouman (1960) originally demonstrated the potentiality of pancreas pieces as a model for the investigation of insulin secretion, the first systematic studies using this preparation were conducted by Coore & Randle (1964). A particular problem with this technique is the interference of an insulin-lytic substance derived from the exocrine tissue. The substance appears to be more abundant in rat pancreas than in rabbit pancreas: it does not directly effect insulin release, and it degrades other small peptides such as glucagon, but not larger peptides such as growth hormone (Coore & Randle, 1964; Malaisse, Malaisse-Lagae, Lacy & Wright, 1967a; Malaisse, Malaisse-Lagae & Mayhew, 1967b; Malaisse, Malaisse-Lagae & Wright, 1967c). Trypsin and chymotrypsin are not likely to be responsible for the lytic activity since they appear to be present in the form of inactive precursors (Ravin, Bernstein & Seligman, 1954; Coore & Randle, 1964), but trypsin inhibitors nevertheless reduce this activity (Trautschold, Werle & Zickgraf-Rudel, 1967; Kassell, 1970). Modifications of the technique to minimize interference by insulin degradation include the use of trypsin inhibitors and/or anti-insulin anti-sera

(Malaisse et al., 1967b, c), prior ligation of the pancreatic ducts to reduce the volume of exocrine tissue (Mjahle & Meyer, 1961) and simultaneous incubation with diaphragm tissue (Bouman, 1960), limitation of the incubation period and selection of the animal species (Coore & Randle, 1964; Hales & Milner, 1968). Preparations in which the exocrine component is entirely eliminated are considered in the following section.

Incubation of isolated islets

A micro-dissection technique for the isolation of intact pancreatic islets was developed by Hellerström (1964). The ease with which the islets may be obtained is dependent on their size and distribution within the pancreas, and the fibrosity of the pancreas. For this reason the technique has been applied to a limited number of animals in which these factors are favourable. Pancreatic duct ligation has been used to facilitate the procedure (Keen, Sells & Jarrett, 1965).

A second technique for the isolation of pancreatic islets involves disruption of the exocrine tissue with the enzyme collagenase and separation of the islets by sedimentation (Moskalewski, 1965; Lacy & Kostianovsky, 1967). The concentration of the enzyme and the time of exposure to the pancreas must be carefully regulated to prevent digestion of the islet capsule and its contents. Identification of the islets may be improved by various coloured dyes (Aleyassine & Gardiner, 1972). Although the collagenase method is more commonly employed than the micro-dissection method, since large numbers of islets may be harvested in a considerably shorter time, there is evidence to

suggest that collagenase may impair normal islet metabolism (Atkins, 1972).

Incubation methods with either pieces of pancreas or isolated islets provide an estimate of the amount of insulin released during the incubation period. They are not suitable for investigations of the dynamic aspects of insulin secretion since rapidly repeated sampling of the incubation medium is not practicable. Techniques applicable to investigations of this kind are described below.

Isolated perfused pancreas

A method for perfusion of the isolated pancreas was first described by Anderson & Long (1947), and subsequently modified by Grodsky and co-workers (1963) and Sussman and co-workers (1966). The perfusion fluid was supplied to the organ through the normal arterial vessels and collected from the hepatic portal vein, and all three groups employed a closed circuit, that is, a circuit in which the perfusate is re-circulated. Problems were encountered in maintaining the constancy of the perfusate, due to the accumulation of metabolites and the removal of substrates; in oxygenating the preparation; in accurately determining the dilution of test substances added, and in replacing fluid withdrawn for sampling. These problems were largely obviated by the introduction of a system in which the perfusion fluid is passed through the pancreas without re-circulation (Curry, Bennett & Grodsky, 1968). This type of system is currently employed by a number of research groups and has been adopted in the present study.

In view of the complex technical aspects of pancreatic

perfusion techniques more simplified procedures for the investigation of dynamic aspects of insulin secretion have been sought.

Perifusion systems

Very recently, systems have been described in which pieces of pancreas (Burr, Stauffacher, Balant, Renold & Grodsky, 1969; Junod, Letarte, Lambert & Stauffacher, 1969) or isolated islets (Idahl, 1972; Lacy, Walker & Fink, 1972; Hoshi & Shreeve, 1973) are suspended in a continuous flow medium based on open circuit pancreatic perfusions. These so-called perifusion systems have proved relatively simple to prepare and manipulate and although they may not simulate physiological conditions as closely as the perfusion systems, the metabolic characteristics of perifused islets have been in general agreement with data obtained using perfusion systems. Perifusion systems have the advantage that several channels may be run in parallel using tissue from the same animal. Thus control and test conditions may be investigated simultaneously. It is envisaged that this technique may be modified for studies of the dynamic properties of individual islet cells.

Culture of islet tissue

Studies of insulin secretion in vitro using the above methods have been of a relatively short-term nature, extending for no longer than a few hours. To enable more prolonged studies, methods are being developed for the culture of islet tissue over periods of several days to several weeks. The endocrine component of the neonatal pancreas continues to proliferate

in vitro, and cultures of foetal pancreas or dispersed islet cells from this source have been demonstrated to retain insulin secretory competence for a number of weeks (Macchi & Blaustein, 1969; Lambert, Blondel, Kanazawa, Orci & Renold, 1972; Chick, Lauris, Flewelling, Andrews & Woodruff, 1973). Isolated islets have also been cultured successfully for 2 weeks (Moskalewski, 1965; Anderson & Hellerström, 1972; Kostianovsky, Lacy, Greider & Still, 1972). The morphological and metabolic properties of islet cells appear to alter during culture and experiments on cultured tissue should take these alterations into account.

Incubation of pieces of pancreas - method

The procedure adopted for in vitro studies of insulin secretion from pieces of rat pancreas was based on the one described by Coore & Randle (1964) with several modifications.

The incubation medium was a bicarbonate buffered salt solution (Gey & Gey, 1936), oxygenated with a gas phase of $O_2 + CO_2$ (95:5), pH 7.4. Bovine serum albumin (fraction V), 2.0 mg/ml (Armour Pharmaceutical Co. Ltd., Eastbourne) was included as a colloid supplement in certain media. Soybean trypsin inhibitor (basic bovine pancreatic trypsin inhibitor, Kunitz) 20.0 μ g/ml (type 1-P, Sigma Chemical Co., London) was also included in certain media to reduce the activity of the exocrine insulin-lytic factor (Malaisse et al., 1967b).

Test incubations were performed in 1 dram glass vials fitted with polythene stoppers (Johnson & Jorgensen Ltd., London). The inner surface of the vials was treated with a

biologically inert silicone liquid (Sigmacote, Sigma Chemical Co.) which forms a thin film on the glass and prevents contact of the contents of the vial with the glass. This precaution reduces the loss of insulin through adsorption to glass (Ferrebbee, Johnson, Mithoefer & Gardella, 1951). Vials were rinsed with the appropriate buffer immediately before use.

Following the recommendations of Malaisse et al. (1967c) the pancreatic tissue was dissected into pieces weighing about 10 mg and test incubations were performed on small groups of pieces (usually 6 pieces in 2 ml of medium). These workers have shown that insulin secretion does not continue to increase linearly with the weight of pancreatic tissue when there is in excess of about 100 mg of tissue in 2 ml of medium. They have also shown that smaller pieces of pancreas tend to yield greater amounts of insulin, which is presumed to reflect the ease of exchange between islets and medium; but the extent of smallness is limited by the likelihood of causing excessive islet disruption. If each piece of pancreas does not serve as its own control (as in the present studies) it is of advantage to use several pieces in each test incubation to reduce the effect of an uneven distribution of islets among the pieces. The above recommendations represent a convenient compromise of these criteria.

The incubation medium was shaken at a gentle and consistent rate throughout the present studies. Gentle shaking (which does not cause physical harm to the tissue) appears to increase the yield of insulin (Hellman, 1970). Shaking of the medium facilitates the dispersion of insulin within it: this

prevents the build-up of insulin around the islets which may reduce the rate of insulin secretion, and guards against the withdrawal of an unrepresentative sample of medium for insulin assay.

Levels of insulin in excess of about 1,000 μ U/ml produce a detectable negative feedback on insulin secretion (Malaisse, Malaisse-Lagae, Lacy & Wright, 1967d; Frerichs, Creutzfeldt & Creutzfeldt, 1968; Loubatières, Mariani & Chapal, 1968; Hahn & Michael, 1970). Such levels of insulin were avoided in the present studies by the experimental design.

Before undergoing test incubations the tissue was preincubated for at least 20 minutes in a low glucose medium (0.5 or 0.6 g/l) to eliminate differences of insulin secretion associated with the terminal glycaemia of the animal. The preincubation period also permits dispersion of the contents of cells that are ruptured during dissection of the pancreas, and especially large amounts of insulin which are released from ruptured islets. Concentrations of glucose below 1.0 g/l produce only a marginal stimulatory effect on insulin secretion (Malaisse et al., 1967c). Hence, preincubation under these conditions preserves the insulin content of the islets.

Insulin secretion was expressed as μ U/mg dried pancreas per incubation period (30 or 60 minutes). The pieces of pancreas were dried on aluminium foil boats for 48 hours at 105°C and weighed to the nearest 0.1 mg.

Several criticisms may be levelled at the present technique. In common with other incubation methods the original

composition of the incubation medium becomes modified during the experiment, due to the uptake of substrates and the liberation of metabolites by the tissue. This is particularly relevant to the preparation of pancreas pieces since the acinar component is in considerable excess of the insular component under study. Furthermore, the acinar tissue has a specialized secretory function and liberates a factor that degrades insulin. The secretion of insulin has been expressed in terms of the total weight of pancreatic tissue. It is appreciated that not necessarily correspond with the amount of islet tissue.

There is a potential advantage of studying islet metabolism in the presence of exocrine tissue. It is possible that the intimate anatomical relationship between the acinar tissue and the insular tissue is consummated with a functional association.

Determination of blood glucose

Numerous methods have been described for the quantitative estimation of glucose in blood (see reviews by Henry, 1966; Natelson, 1971). Two general categories may be distinguished:

- (i) Methods based on the reducing properties of glucose, which are usually quantified through a primary or secondary colorimetric reaction. These methods are frequently susceptible to interference from other reducing substances present in blood and may therefore provide an over-estimation of glucose.
- (ii) Methods based on the enzymatic breakdown of glucose either by glucose oxidase (glucose dehydrogenase) or by

hexokinase and glucose-6-phosphate dehydrogenase. Similarly, quantitation is achieved by coupling the system with a colorimetric reaction. Since the enzymes are virtually specific for glucose these methods have been deemed to measure "true" glucose, but recent evidence suggests that certain humoral factors may interfere with the activity of the enzymes and with the quantitation of the product of enzymatic action (Goodwin, 1970; Wright, Rainwater & Tolle, 1971; Meites & Sauiel-Banrey, 1973).

The various procedures have been modified frequently to improve specificity and sensitivity, and to simplify methodology. Several methods are commercially available in kit form and all of the principle methods have been automated.

Recently the glucose oxidase reaction has been adapted for the instant determination of glucose in blood. The technique relies on an oxygen electrode to quantify the amount of oxygen utilized in the reaction (Kadish, Little & Sternberg, 1968; Bessman & Schultz, 1972). This development offers the prospect of automated insulin therapy for diabetic patients: a device could be implanted into the body which would continuously monitor blood glucose and would simultaneously meter out the required amount of insulin. An almost immediate semi-quantitative estimation of blood glucose, based on the glucose oxidase reaction is already in routine clinical use (Dextrostix, Ames Company, Slough; Drury, Sweeney & UaConaill, 1972).

In the present study blood glucose has been estimated by the ferricyanide reduction method, using a Technicon autoanalyzer (Salway, 1969). The method is based on the

original procedure of Hoffman (1934) and follows the standard adaptation of this procedure to the autoanalyzer (Method sheet N-2b, Technicon Instruments Company Ltd., Chertsey) with minor modifications.

Freshly collected samples of blood were diluted 1:5 in a preservative-anticoagulant solution (0.1% (w/v) sodium fluoride containing heparin 60 U/ml) and analysed as soon as possible. The analysis essentially involves dilution of an aliquot of the blood-preservative-anticoagulant mixture with saline, followed by dialysis at 37°C into a solution of alkaline potassium ferricyanide (saline; 0.025% (w/v) potassium ferricyanide; 2.0% (w/v) sodium carbonate) which is heated to 95°C. The yellow ferricyanide solution is partially reduced to colourless ferrocyanide by the glucose, and the extent of decolouration is determined using a 15 mm flow-cell colorimeter at 420 nm. Unknown amounts of glucose were quantified with reference to a standard curve which was constructed by plotting known concentrations of glucose (0, 50, 100, 200, 300, and 350 mg/100 ml) against their respective recorder peak height tracings (equivalent to optical transmission). A linear graph was obtained for values of glucose up to 350 mg/100 ml) and all samples analyzed were within this limit.

The procedure is not specific for glucose since other reducing substances in the blood may contribute to the ferricyanide reduction. These substances are principally other reducing sugars, but creatinine, glutathione, ascorbic acid and uric acid may also be involved. For this reason the term "blood sugar" is considered more appropriate than "blood

glucose". A proportion of the non-glucose reducing substances is removed by dialysis, but the amount remaining has been variously estimated to account for between 5 and 20 mg/100 ml. The percentage error is greater at low levels of glucose, but the variation of absolute error at different glucose concentrations and in different individuals is considered to be negligible.

Histological technique

Histological examination of the islets of Langerhans was carried out in conjunction with certain metabolic studies. Pancreatic tissue was fixed for 24 hours in a solution of 10% (w/v) formal-saline, dehydrated through graded alcohols, cleared in chloroform and embedded in paraffin wax. Serial sections of 5 μ m thickness were cut using a rotary microtome (Baird & Tatlock Ltd., London) and mounted individually. Consecutive slides were processed by one of the following staining techniques: haematoxylin and eosin (H.E.) (Drury & Wallington, 1967, p. 129) to demonstrate general morphology; Gomori's aldehyde fuchsin (A.F.) (Gomori, 1941) counterstained with 2% (w/v) aqueous light green, (after Drury & Wallington, 1967, p. 184) to demonstrate beta cells; and silver impregnation (Grimelius, 1964, 1968a, 1968b) to demonstrate alpha (A_2) cells. Islets for photography were chosen at random from representative sections (Ektachrome EHB 135-20, Kodak, London; Zeiss MK.I photomicroscope, Carl Zeiss, Degenhardt & Company Ltd., London).

Chemicals

All reagents used were of analytical grade and solutions

were prepared with double distilled water unless otherwise stated.

Cleaning of glassware

Glassware was rinsed thoroughly in tap water and immersed overnight in a detergent solution (Lab-brite, The British Hydrological Corporation, London). The detergent was eliminated with several rinses in tap water, then in distilled water and finally in double distilled water before drying in an oven at 45°C.

Radio-isotopes

Special precautions in accordance with the code of practice recommended by the Department of Employment and Productivity (1968) and the University of Aston (1969) were observed when working with radioisotopes. Contaminated glassware was immersed for a week in a solution of Decon 75 (Medical-Pharmaceutical Developments Ltd., Brighton) prior to the normal washing procedure.

Statistical Method

Where appropriate, the mean (\bar{m}) and the standard error of the mean (sem) of a group of data were calculated. Different groups of data were compared using Student's t -test, and a statistically significant difference was accepted for probability levels of $p < 0.05$ (Bishop, 1966). Calculations were performed with the aid of a desk computer (Olivetti Programma 101, British Olivetti Ltd., London).

Figure 1 Cylindrical plastic restrainer

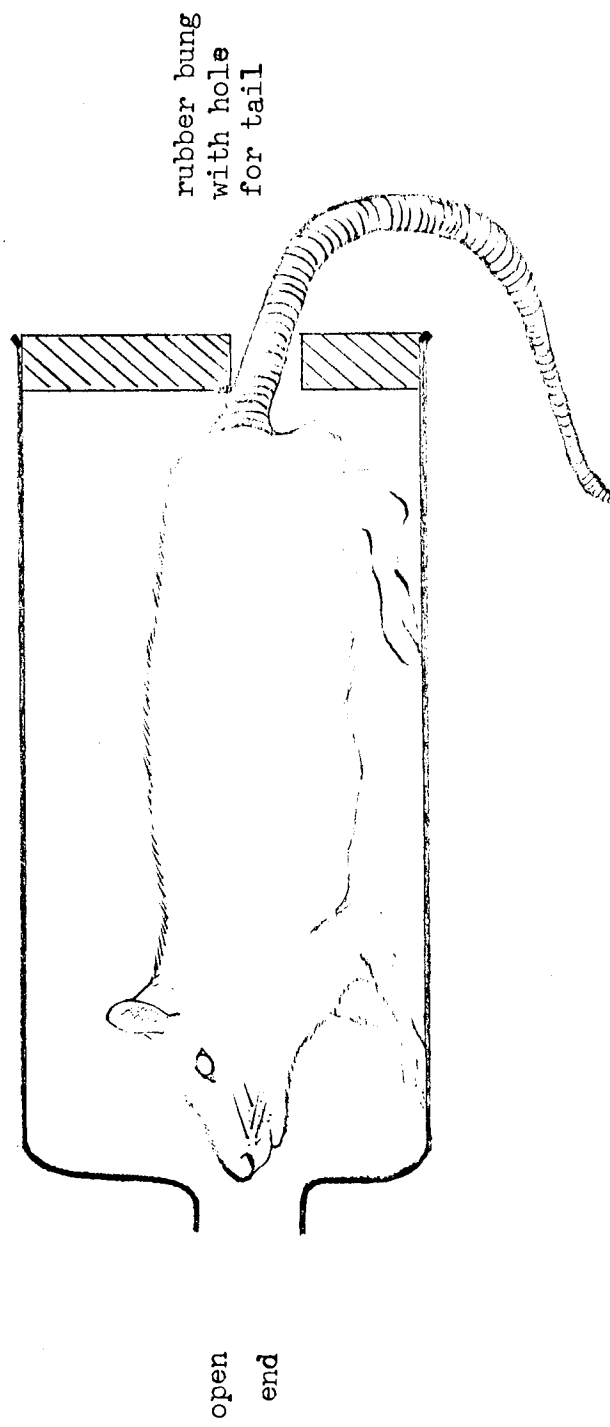


Figure 2 Analysis of intraperitoneal glucose tolerance tests as a function of the area beneath the glucose curve, termed the glucose tolerance area ($ipGT_A$). For explanation see text

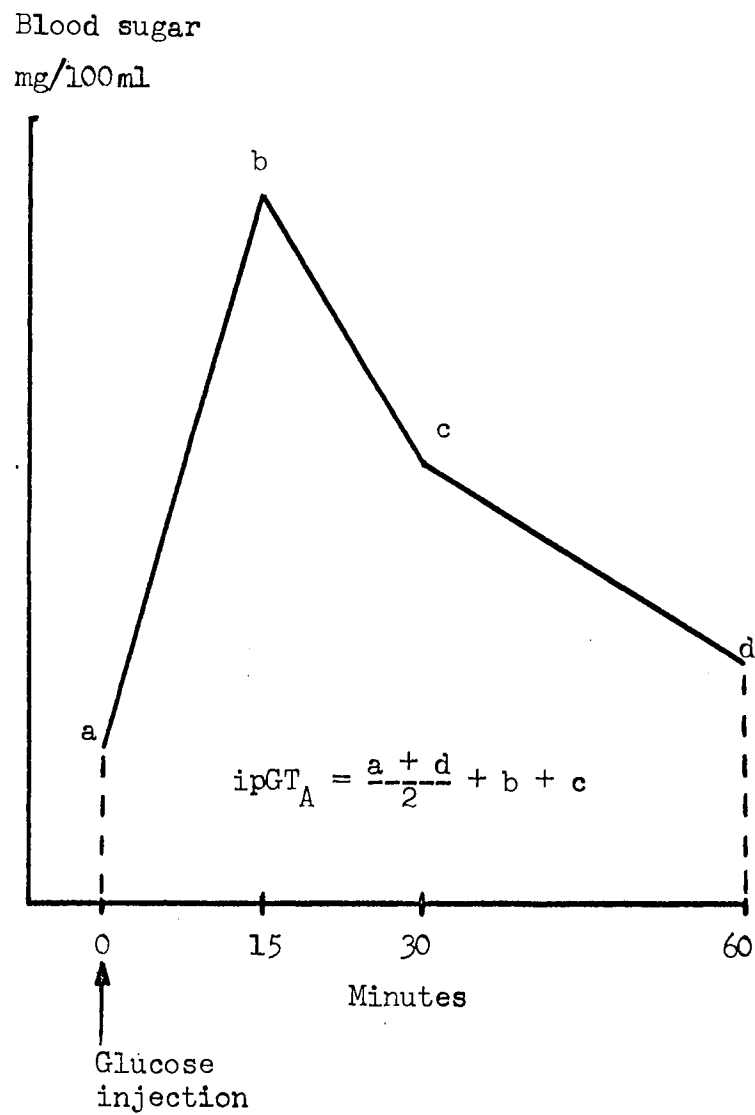
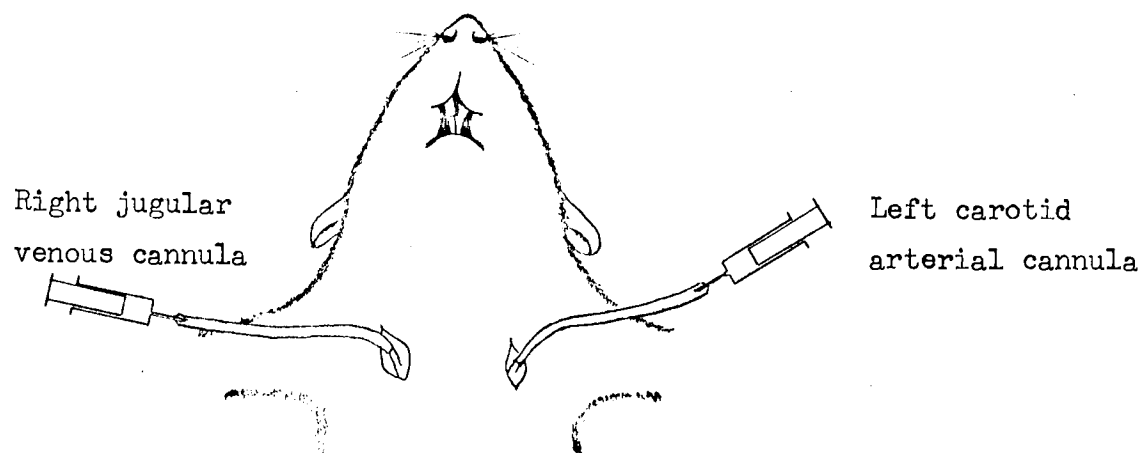


Figure 3 Preparation of animal for intravenous
glucose tolerance tests



CHAPTER 3

RADIOIMMUNOASSAY OF INSULIN

RADIOIMMUNOASSAY OF INSULIN^{*1}

A variety of procedures have been described for the quantitative estimation of insulin in body fluids. Early attempts were based upon the biological activity of the hormone, but these have been largely superseded by immunological methods of assay, in particular radioimmunoassay methods, which have proved to be highly sensitive and specific.

Bioassay

The original bioassay procedures were performed in vivo and depended on the ability of the hormone to produce hypoglycaemia in rabbits and hypoglycaemic convulsions in mice. Subsequent in vitro methods were developed which involved the measurement of glucose uptake and glycogen deposition by isolated hemidiaphragms or epididymal fat pads of rats and mice (see reviews by Randle & Taylor, 1960; Stewart, 1960; Vallance-Owen & Wright, 1960). However, these methods were not satisfactory on several accounts. Sensitivity was not always adequate to allow the determination of normal levels of insulin in blood: specificity was poor, since the inherent variability of tissues and the effects of other biologically active constituents of the blood were incorporated in the assay system, and the procedures were generally expensive, complicated to perform and lacking in reproducibility.

Footnote ^{*1} = A glossary of terms used in this chapter is given on page 73

Immunoassay

Immunological methods of hormone assay are based on the interaction of the hormone with a specific antibody to form a hormone-antibody complex.

Insulin Antibodies

Antibodies to both endogenous and exogenous insulin have been detected in the γ -globulin fraction of animal and human sera (see review by Wright, 1965). For the purpose of immunoassay, antibodies are conveniently raised by immunizing a selected species with insulin from a different species. Insulin antibodies derived in this way vary widely in their neutralizing potencies; they do not appear to be species specific although the extent to which they cross-react with heterologous forms of insulin varies greatly (Berson & Yalow, 1959; Yalow & Berson, 1960; Berson & Yalow, 1963; Berson & Yalow, 1964; Berson, Yalow, Glick & Roth, 1964). Antisera are selected for the particular qualities required - principally high specificity and avidity for the hormone that is to be assayed (Hurn & Landon, 1971). The most potent anti-insulin serum has been raised in guinea pigs (Wright, Kreisberg, Halpern & Dolkart, 1962) and this source of antibody is employed in all current insulin radioimmunoassays.

Non-radiological immunoassays

Insulin immunoassay systems are conveniently classified into those which do not employ radioisotopes and those which do employ radioisotopes (radioimmunoassay). Three procedures have been described that fall into the former category.

Arquilla and co-workers (1956, 1960) exploited the ability of insulin antibodies to agglutinate insulin-sensitized erythrocytes. An estimate of the amount of insulin associated with an excess of anti-insulin serum was derived from the extent to which the anti-insulin serum subsequently inhibited the agglutination of insulin-sensitized erythrocytes. Loveless (1956) determined hyperinsulinism in certain subjects by the wealing reponse that occurred when local areas of skin were sensitized to insulin by an intracutaneous injection of human anti-insulin serum. Jones & Cunliffe (1961) and Birkinshaw, Randall & Risdall (1962) reported a procedure based on the observation of Moloney & Aprile (1959) that insulin antibodies produce precipitin bands by the gel diffusion technique of Ouchterlony (1948). A measure of the amount of insulin associated with the antibodies was derived from the position, shape and intensity of these bands. However, these methods were not sufficiently sensitive for the determination of normal insulin levels in biological fluids and they are no longer used.

Radioimmunoassay

The concentration of insulin in biological fluids is now determined almost universally by radioimmunological methods. In comparison with the alternative procedures for insulin assay (described above), radioimmunological methods confer advantages of specificity, sensitivity and reproducibility. In addition, they are relatively inexpensive and straightforward to perform. The concept of radioimmunoassay was pioneered by Doctors Rosalyn Yalow and the late Solomon Berson, who, in a series of outstanding reports dating from 1956 developed a radioimmunoassay for insulin (Yalow & Berson, 1960). Subsequent developments

in this branch of endocrinology have been rapid and extensive, and numerous reviews have appeared (- most recently by Skelley, Brown & Besch, 1973). The present review is necessarily limited to a consideration of the basic principles and fundamental modifications of insulin radioimmunoassay systems.

Principles of radioimmunoassay

Radioimmunoassay is based on the principle of isotopic dilution in the presence of specific antibodies. This principle depends on the competition between radioactively-labelled hormone and unlabelled hormone for the binding sites of the antibody. Increasing amounts of unlabelled hormone in the test fluid cause proportionate decreases in the attachment of labelled hormone to the antibody. Thus the level of radioactivity measured in the hormone-antibody complex is related in an inverse manner to the amount of unlabelled hormone in the original test fluid, and the latter value may be accurately quantified by reference to a standard curve.

In practical terms this means that the unlabelled insulin (standard or unknown) and a relatively small amount of labelled insulin are incubated with a small amount of anti-insulin serum. The labelled and unlabelled hormone thus compete for the antibody binding sites and the binding of labelled insulin decreases in a non-linear fashion as the amount of unlabelled insulin is increased. As an alternative to the "competitive" system, Wright and co-workers (1968, 1969, 1971) have developed a "back-titration" procedure in which an excess of antibody is preincubated with unlabelled insulin, and the partially

neutralized serum is subsequently incubated with an excess of insulin, part or all of which may be labelled. In this system binding of labelled insulin decreases linearly as the amount of preincubated insulin is raised. The "back-titration" method offers the practical advantage of minimal critical dilution procedures.

Criteria of validation

Several criteria must be satisfied by an ideal radioimmunoassay system. The unknown, standard and labelled hormones should be immunologically identical with respect to the specific antibody concerned; the system should be devoid of other (contaminant) immunoreactive material, and non-specific factors such as pH, ionic environment and temperature should be standardized at the optimum level. Although the sensitivity of the assay may be diminished if the labelled and unlabelled hormones react differently, the specificity of the assay is not dependent on the immunological identity of the labelled hormone, since the system is essentially comparing the amount of immunoreactive unknown with the amount of immunoreactivity of the standards. In order to obtain an "absolute" measurement of an unknown hormone it is necessary for the unknown and standard hormones to react identically with the antibody. However, a "relative" value can be derived using a standard hormone that is immunologically similar, though not identical to the unknown.

The immunological reactivity of insulin is not necessarily correlated with its biological activity. It is therefore important to distinguish between the results obtained using immunoassay and bioassay methods. The former methods measure

"immunoreactive insulin" (IRI) and the latter methods measure "insulin-like activity" (ILA). Insulin-like activity includes the effects of insulin and other uncharacterized proteins present in blood which produce similar effects to insulin in bioassay systems (see review by Yalow & Berson, 1964, pp. 595-609). These uncharacterized proteins, the effects of which are not eliminated by the presence of excess insulin antibodies do not appear to originate from the pancreas (Pottenbarger, De Los Montero Mena & Steinke, 1970). Thus, insulin-like activity may be regarded as an over-estimation of circulating insulin levels.

Radioimmunological estimations of circulating insulin levels should be interpreted with extreme caution. The biological efficiency of insulin requires almost complete structural integrity of the molecule, but the requirements for immunological competence are less specific. For example, minor structural changes in the insulin molecule which profoundly alter or abolish biological activity (Nicol, 1960) may not perceptibly affect immunoreactivity (Berson & Yalow, 1963). It is therefore possible that biologically "lame" insulins (Samols, 1965; Hutt, Horton, & Lebovitz, 1967), fragments of insulin molecules which have retained immunoreactivity but are biologically inert, and proinsulin (Steiner, Hallund, Rubenstein, Cho & Bayliss, 1968; Varandani, 1968; Kerp, Kasemium & Steinhilber, 1971) may contribute to the radioimmunological estimation of insulin in blood. Furthermore, circulating antibodies may also interfere with the immunoassay of insulin. It is envisaged that the amount of proinsulin which is measured by insulin radioimmunoassays will be clarified by the introduction of assays

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specific for this precursor molecule (Markussen, Heding, Jorgensen & Sundby, 1971; Miles, 1971). Insulin radioimmunoassays are believed to measure all of the biologically active insulin in blood (Yalow & Berson, 1964; Taylor, 1967), but although the specificity of these systems is considerably greater than the specificity of bioassay systems, an over-estimation of the absolute concentration of insulin still appears likely.

Separation of antibody-bound and free labelled insulin

There are several types of "competitive" insulin radioimmunoassay systems in current use. These systems differ from one another in one major respect - the methods which they employ to separate antibody-bound from free labelled insulin in the final reaction mixture (table 3). The values obtained in different laboratories using the various separation techniques appear to be remarkably uniform, and experiments in which two or more different separation techniques have been directly compared substantiate this observation (Samols & Bilkus, 1964; Rosselin, Assan, Yalow & Berson, 1966; Ashford, Campbell, Davidson, Fisher, Haist, Lacey, Lin, Martin, Morley, Rastogi & Storvik, 1969; Cotes, 1969; Taljedal & Wold, 1970; Buchanan & McCarroll, 1971; Logsdan, 1971, Palmieri, Yalow & Berson, 1971; Raptis, 1971).

Radioactively-labelled insulin

The amount of radioactively-labelled insulin used in the "competitive" assay system must be kept to a minimum so that the combining sites of the antibody are not saturated by this alone. In order to detect the minute quantity of tracer which becomes

attached to the antibody, a radioisotopic label of high specific activity is required (unless an inconveniently large volume of incubate is used). At the same time, the labelled hormone must retain its native antigenicity. Consistent with these requirements it has been found convenient to label the tyrosine residues with ^{131}I or ^{125}I by substitution of the hydrogen atoms in the aromatic ring. The specific activity of the labelled hormone increases with the extent of radioiodination, but since there is a concomitant reduction of immunoreactivity of the hormone, the extent of radioisotopic labelling must be minimal. An arbitrary maximum substitution of an average of 1 atom of radioisotope per molecule of insulin has been suggested because alterations of immunoreactivity have been detected at higher levels (Ooms & Arquilla, 1966; Freedlender, 1969). The levels of substitution employed in current radioimmunoassays are considerably below this limit. The isotopic abundance and the counting efficiency of the radioisotope also affect the specific activity of the labelled hormone. Although ^{131}I has a theoretical 7 fold advantage over ^{125}I because of its shorter half-life, this advantage cannot be realized. Iodine-125 is available virtually in a carrier-free state, but ^{131}I rarely exceeds an isotopic abundance of 30%. In addition, the counting efficiency of ^{125}I is considerably greater than that of ^{131}I . Early radioimmunoassays used ^{131}I -labelled insulin, prepared by methods available at that time (McFarlane, 1958; Yalow & Berson, 1960; Izzo, Bale, Izzo & Roncone, 1964). However, since highly purified ^{125}I has subsequently become available, and the initial problems of iodinating with this isotope have been resolved, the

advantages of ^{125}I may be realized, and this isotope is currently preferred. Radioiodination is now most commonly effected by methods based on the chloramine-T reaction (Greenwood, Hunter & Glover, 1963).

Solid phase assay

Prominent among recent developments in radioimmunoassay techniques is the introduction of a "solid phase" assay system. This involves the attachment of antibody to an insoluble material such as dextran (by chemical covalent binding), polystyrene discs or the inner surface of polystyrene tubes (by physical adsorption) (Catt, Niall & Tregear, 1967; Catt & Tregear, 1967; Wide, Axen & Porath, 1967). The advantage of the solid phase system resides in the separation of antibody-bound and free labelled hormone, which may be accomplished by simple filtration or centrifugation, or aspiration of coated tubes. Solid phase procedures were originally applied to the assay of growth hormone, but their application to the assay of insulin has subsequently been reported. Some groups claim only limited success using antibody coated tubes (Catt, Tregear & Burger, 1970; Albano, Ekins, Maritz & Turner, 1972; Miedema, Boelhouwer & Otten, 1972) but Colt, Becker & Quatrone (1971) and Bates & Garrison (1971) have described a sensitive and very rapid assay using this method. Very recently a sensitive solid phase insulin radioimmunoassay that utilizes dextran-bound antibodies has become commercially available (Pharmacia AB 1972), and the optimal conditions for this assay have been evaluated by Velasco, Oppermann & Camerino-Davalos (1973).

Immunoradiometric assay

A novel technique for the assay of insulin, termed an

"immunoradiometric" assay has been developed by Miles & Hales (1968 a, b). This assay incorporates certain features of the solid phase system, but its unique property is the use of radioiodinated antibody. The labelled antibody is incubated in excess with standard or unknown hormone, and the untreated portion is removed by incubation with excess insulin attached to a cellulose solid phase. Thus, the unreacted antibody becomes attached to the cellulose-bound (insulin-excess unreacted antibody) complex and is separated by centrifugation. The supernatant, which contains the original hormone bound to the labelled antibody is then counted - the amount of radioactivity being directly proportional to the quantity of insulin. This method offers several advantages. It eliminates the use of a labelled hormone, which, on account of possible damage during labelling, has been cited as a source of error in competitive radioimmunoassay systems. The immunoradiometric assay operates at low background, because in the absence of a labelled hormone there can be no labelled derivatives or hormone fragments formed, and the assay provides a relatively stable and uniform product for counting. Together with the "back-titration" procedure, the immunoradiometric assay also offers a reaction which favours the complete interaction of antigen and antibody, and involves a minimum of critical dilution steps.

Application of radioimmunoassay

Insulin radioimmunoassay techniques have been widely applied in medicine and related disciplines. The comparative ease with which large numbers of samples may be assayed by these

techniques has enabled routine clinical analyses of insulin in blood. This has greatly facilitated the diagnosis of disorders that involve abnormal insulin secretion or metabolism. Radio-immunoassays have also been developed for the determination of insulin in urine (Jorgensen, 1966; Chamberlain & Stimmeler, 1967; Rubenstein, Lowy & Fraser, 1967; Rubenstein, Lowy, Welborn & Fraser, 1967; Fraser, 1969; Orskov & Johansen, 1972). These assays have proved valuable for monitoring the amounts of insulin available to the body during long periods (e.g. 24 hours) and for obtaining information on the renal handling of insulin. Insulin radioimmunoassays have been extensively exploited as a research tool, particularly towards an understanding of the dynamic aspects of insulin secretion and their control. Associated with the requirements of routine clinical analyses and specific research programmes, numerous minor modifications to the various assay systems have been described. Such modifications have generally been concerned with simplifying methodology or improving specificity, sensitivity, accuracy or reproducibility.

Radioimmunoassay used in present study.

The insulin radioimmunoassay procedure adopted in the present study is based on the double antibody method of Hales & Randle (1963 a, b). Characteristic of the double antibody method is the use of a second antibody - an anti - γ -globulin serum - to precipitate the insulin-antibody complex. The process is termed immunoprecipitation. In this way the antibody-bound insulin may be separated from the free labelled insulin present in the final reaction mixture. This separation technique was derived from the demonstration by Sköm & Talmage (1958) that insulin, bound in vitro by serum obtained from insulin resistant patients can be

precipitated by an antibody to human γ -globulin.

The assay procedure comprises the initial reaction of standard or unknown insulin with anti-insulin serum (first antibody) obtained from immunized guinea pigs to form a soluble insulin-antibody complex. Radioiodinated insulin is then introduced and allowed to react with the antibody until equilibrium is reached. Under ideal conditions, the resulting complex comprises labelled and unlabelled insulin, bound in proportion to their molar concentrations in the reaction mixture. The insulin-antibody complex is precipitated by an anti-guinea pig γ -globulin serum (second antibody) obtained from immunized rabbits, and sedimented by centrifugation. The free labelled insulin is then removed by decantation. The radioactivity of the precipitate is counted and unknown amounts of insulin are quantified by reference to a standard curve.

Attention has been drawn to the possibility that γ -globulin present in a sample of plasma undergoing assay may interfere with the precipitation of the insulin-antibody complex by cross-reacting with the precipitating rabbit antiserum, unless very high concentrations of the latter are used. However, since insulin antibody that has been precipitated with anti- γ -globulin serum is still capable of reacting with insulin, Hales & Randle (1963a) were able to obviate this possibility by precipitating the insulin antibody before the plasma sample was added.

It has also been observed (Hales & Randle, 1963a) that the sensitivity of the assay is improved when the antibody and the unlabelled hormone are reacted together before the labelled component is added. Hence, this procedure has been followed in the present system.

Emphasis is laid on the fact that the radioimmunoassay of rat insulin described below provides only a "relative" estimation of the IRI values, since anti-porcine insulin serum and standard human insulin have been utilized. Thus the IRI values for rat insulin reported in this thesis should be strictly considered as human insulin equivalents.

Reagents

Buffer solutions were prepared with deionized water ('Elgastat', Elga Products Ltd.), because the quality of ordinary distilled water is inconsistent and may give rise to variations in the amount of insulin bound to the antibody precipitate.

Buffer A, a phosphate buffer (40 mmol, pH 7.4) containing bovine serum albumin (0.51% w/v) and sodium ethylmercurithiosalicylate (thiomersalate) (0.6 mmol) was used to dilute the iodinated insulin. The buffer was prepared by dissolving 5.1g bovine serum albumin, 6.2g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 0.25g thiomersalate in a little under a litre of deionized water. The solution was adjusted to pH 7.4 with aqueous NaOH (about 5 ml of a 2 mol solution) and diluted with deionized water to a final volume of 1 litre. The pH was checked and readjusted to pH 7.4 with further NaOH if necessary.

Buffer B is an isotonic phosphate buffer comprised of buffer A plus 0.9% (w/v) NaCl. This buffer was used to dissolve and dilute the standard insulin and to dilute assay samples when this was required.

Buffer C is a high protein buffer consisting of buffer A and horse serum in equal proportion used for washing the antibody precipitate. The horse serum (no. 5, Wellcome Reagents Ltd.,

Beckenham) was used immediately after purchase and rarely required filtering.

The insulin antibody and the standard and labelled insulin preparations were obtained from The Radiochemical Centre, Amersham, in a commercially available insulin immunoassay kit (code IM 39).

The antibody was supplied in a preprecipitated form (insulin binding reagent), having been prepared for The Radiochemical Centre by Wellcome Reagents Ltd. Antiserum to porcine insulin was raised by immunization of guinea pigs and antiserum to guinea pig- γ -globulin was raised by immunizing rabbits. Porcine insulin differs from human insulin only in the C-terminal amino acid residue of the B chain (fig.4). It appears that antibodies are not directed against this residue and must therefore react with a region of the porcine insulin molecule which has the same primary structure, but not necessarily the same three-dimensional configuration as the corresponding region of the human insulin molecule (Berson et al., 1964). Further, human, bovine and porcine insulins are virtually undistinguished by anti-pork insulin serum (Yalow & Berson, 1963) and for this reason anti-pork insulin serum is generally accepted as a suitable less-expensive alternative to anti-human insulin serum in radioimmunoassays. The anti-insulin serum was diluted in buffer A containing ethylenediaminetetraacetic acid (EDTA) so that the working solution consisted of a 1:16,000 dilution of antibody in 0.03 mol EDTA. The antibody was precipitated by incubation with a predetermined amount of guinea

pig- γ -globulin antibody at 4°C for 18 hours and then freeze dried. This material was stored at 4°C and reconstructed with deionized water (8 ml per vial as supplied). The working solution of insulin binding reagent will bind labelled bovine insulin up to a concentration of approximately 25 μ U/ml, which is approximately 40% of the amount of tracer added to the system (Radiochemical Centre, 1969).

Purified human insulin used as standard and purified bovine insulin used as tracer were also prepared for The Radiochemical Centre by Wellcome Reagents Ltd. Crystalline human insulin was dissolved in buffer B and solutions containing 250, 100, 50, 20 and 10 μ U/ml were prepared by serial dilution.

As mentioned previously, an assay is not invalidated if the labelled hormone is not immunologically identical to the standard hormone. Bovine insulin was employed as the tracer hormone because it is more readily available than human insulin and shows a very similar affinity for anti-porcine insulin serum to the human standard (Yalow & Berson, 1963). The purified bovine preparation (potency 24.3 iu/mg) was radioiodinated with ^{125}I by a modification of McFarlane's (1958) iodine monochloride method, and unbound iodine was removed by gel filtration (Radiochemical Centre, 1973). The extent of substitution was always less than an average of 0.5 atoms of ^{125}I per molecule of insulin. The hormone was dissolved in buffer A at a concentration of 0.02 μ g/ml and was supplied with a minimum specific activity of 50 μ Ci/mg. This stock solution was stored at -10°C. A working solution containing 62.5 μ U/ml of insulin (250 pg/0.1 ml aliquot) was prepared by diluting the stock solution 1:8 (v/v).

in buffer A.

Buffers and working solutions of insulin binding reagent, standard and labelled hormones were prepared freshly for each assay.

Procedure

The complete procedure was performed in polystyrene tubes, 10mm diameter and 64mm long, fitted with polythene stoppers (Hopkin & Williams Ltd., Romford). All reactants were added in aliquots of 100 μ l using an Eppendorf Marburg micropipette equipped with interchangeable plastic tips (A. V. Howe & Company Ltd., London). All bench procedures were performed in an ice bath to maintain the temperature of the reactants at approximately 4°C, for reasons described below.

The essential features of the assay are illustrated in the flow diagram (fig.5). Unlabelled insulin (standard or assay sample) was initially reacted with the insulin binding reagent (precipitated insulin antibody). Duplicates or triplicates of the same sample were determined in certain experiments to improve accuracy. Triplicate sets of human insulin standards (10, 20, 50, 100 and 250 μ U/ml respectively), zero and blank tubes were included in each assay. The zero tubes contained buffer B in place of the unlabelled insulin in order to evaluate the amount of radioactivity of the insulin-antibody complex in the absence of unlabelled insulin. The blank (or control) tubes contained buffer A in place of the insulin binding reagent in order to evaluate the washing procedure of the antibody precipitate. The initial reactants were mixed using a vortex mixer ('Whirlimixer',

Fisons Scientific Apparatus Ltd., Loughborough) and incubated in a refrigerator at 4°C for 12 hours. The system was maintained at 4°C because Berson & Yalow (1959) have shown that the equilibrium constant of the antigen-antibody reaction is increased as the temperature is lowered. The precise nature of the reaction between hormone and antibody is uncertain, although many authorities believe that the final hormone-antibody complex represents a dynamic equilibrium between the reactants. During the initial (12 hour) incubation, the reaction between the unlabelled hormone and the antibody appears to approach, but not to achieve equilibrium (Wellcome Research Laboratories, 1973). In the interests of reproducibility, an initial incubation time of 12 hours was consistently observed. Although the sensitivity of the assay is improved when the unlabelled hormone is reacted with the antibody prior to the addition of the labelled component (Hales & Randle, 1963a) the reason for this is unestablished.

Following the initial incubation, a 100 µl aliquot of tracer hormone, comprising 250 pg of ¹²⁵I-labelled bovine insulin was introduced, and the reactants were vortex mixed and incubated for 24 hours at 4°C. Triplicate samples of labelled insulin were also added to empty tubes, and retained at 4°C without further additions. These tubes were used to determine the 'total count' of tracer hormone added. At the end of the second (24 hour) incubation, the interaction of the labelled and unlabelled hormones with the antibody is substantially completed (Wellcome Research Laboratories, 1973). Since longer periods of incubation are associated with only

marginal extensions of the hormone-antibody reaction, and may be accompanied by damage to the hormones, a second incubation period of only 24 hours was always employed.

The reaction of hormone and antibody is assumed to obey the law of Mass Action (Berson & Yalow, 1959). Based on this assumption, a mathematical appreciation of the kinetic aspects of the reaction for an ideal system can be derived (Berson & Yalow, 1964; Yalow & Berson, 1968; Ekins, Newman & O'Riordan, 1968). With certain modifications this may be applied to the present system in which the standard, unknown and unlabelled hormones possess different affinities for the antibody (Ekins et al., 1968). In an ideal system the labelled and unlabelled hormones occupy binding sites of the antibody in proportion to their molar concentrations, but in the present system binding is also dependent upon the different hormone-antibody reaction energies.

After the second incubation period the precipitated antibody-bound insulin and the free labelled insulin were separated by a technique similar to that described by Morgan & Lazarow, (1963), Quabbe, (1969) and Czerniak, Chlebowski, Kulcar & Boruchowski, (1970). The antibody precipitate was sedimented by centrifugation for 1 hour at 4°C using an MSE Mistral 4L refrigerated centrifuge (Measuring and Scientific Equipment, Ltd., London) at 1,500 rev/min. (The carrying capacity of the centrifuge limited the maximum number of tubes in a single assay to 96). The supernatant was decanted off: the tubes were everted and drained, and the final drop of supernatant on the lip of the tube was aspirated with a pasteur pipette. The precipitate

remaining in the tube was washed with 1 ml of buffer C at 4°C and the contents of the tube were vortex mixed. The sequence of centrifugation, decantation and washing was repeated twice to ensure the removal of all free insulin. After the third washing each tube was recentrifuged, the supernatant was decanted and the precipitate was allowed to dry at room temperature for 12 hours.

The radioactivity of the precipitate was counted during a period of 10 minutes using a well-type crystal scintillation counter, Packard 3001 Autogamma Spectrometer (Hewlett-Packard Ltd., Slough). The counts were corrected for background and decay and duplicate or triplicate determinations were averaged - any grossly aberrant counts being rejected. The final values were converted to counts per minute. The calculations were performed using a desk computer, Olivetti Programma 101 (British Olivetti Ltd., London) with a specially prepared program (fig.6).

The standard curve was constructed as a semi-logarithmic plot to obtain a linear expression, that is, the log of the insulin concentration was plotted against the respective averaged corrected counts per minute. A typical standard curve is illustrated in figure 7. Since linearity was not maintained for concentrations of insulin greater than 250 $\mu\text{U}/\text{ml}$ this value was the highest standard normally used. The region of greatest discrimination occurs for values between 10 and 100 $\mu\text{U}/\text{ml}$, and plasma IRI values were mostly within this range. Samples of incubation media that were expected to contain insulin in excess of 250 $\mu\text{U}/\text{ml}$ were diluted 1:10 in buffer B (chapter 9). Unknown

values were quantified by determining the concentration of insulin on the ordinate that corresponded with the corrected counts per minute on the abscissa.

Indications of a satisfactory assay were: good agreement between replicates; an adequate washing procedure (blank tubes containing less than 5% of the 'total count'); and the expected binding of tracer and antibody in the absence of unlabelled insulin (zero tubes containing approximately 40% of the total count).

This assay system offers several advantages. The antibody-bound and the free labelled insulin are separated by a simple and effective procedure. Reagents of consistently high quality are readily available and the optimal conditions for the assay have been extensively examined (Radiochemical Centre, 1969; Wellcome Research Laboratories, 1973). The complete assay, including counting is performed in a single disposable reaction vessel. The polystyrene immunoassay tubes used tend to bind antibodies by physical adsorption (Catt & Tregear, 1967), which facilitates retention of the antibody-precipitate in the tube during the separation procedure.

Sources of error

Various likely sources of error are inherent in the present assay system. Human error in the experimental manipulation of the assay, construction of the standard curve and extrapolation of unknown values from the standard curve has been minimized wherever possible, as described in the previous sections of this chapter.

Interference of the assay of insulin in blood may occur due to complement. Morgan & Lazarow (1963) described a double antibody system in which an excess of rabbit anti-guinea pig- γ -globulin serum was introduced to precipitate the insulin-antibody complex. The insulin values derived using this assay were generally high, and occasionally spuriously elevated in comparison with values yielded by current assays. This situation was attributed to complement - a constituent of the test serum (or plasma) which inhibits the precipitation reaction, on occasions very effectively (Morgan, Sorenson & Lazarow, 1964; Soeldner & Stone, 1965; Welborn & Fraser, 1965). Cross-reactivity of the test serum with the precipitating antiserum was considered the most likely cause. However, the double antibody method of Hales & Randle (1963) which utilizes a preprecipitated insulin antibody was also prone to give occasionally very high insulin values (Sheldon & Taylor, 1965), suggesting that other components of complement might be interfering with this method. The effects of complement may be overcome by heat, optimal concentrations of reactants, extended incubation times, dilution of the test sample and addition of a chelating agent such as EDTA (Morgan et al., 1964; Soeldner & Stone, 1965; Sheldon & Taylor, 1965; Welborn & Fraser, 1965; Grant, 1963). In order to eliminate the influence of complement in the present assay 0.03 mol EDTA has been included in the reaction mixture. It has been suggested that EDTA inactivates the responsible components of complement by complexing calcium (Taylor, 1967).

The determinations of insulin in blood reported in this thesis were performed in plasma which contained a minimal quantity of

heparin (specified in the appropriate chapters). It has been suggested that the presence of heparin is advantageous since it exerts an anti-complementary effect similar to that produced by EDTA (Soeldner & Stone, 1965; Welborn & Fraser, 1965). However, Henderson (1970) found consistently higher insulin values in heparin-plasma than in serum. During the last three years several conflicting reports concerning the effects of heparin on insulin radioimmunoassays have appeared (Spellacy & Buhi, 1971; Orosz, Michael & Ziegler, 1971; Henderson, 1971; Aynsley-Green & Alberti, 1972; Feldman & Quickel, 1973; Orosz, 1973; Thorell & Lanner, 1973). It seems likely that methodological differences of assay technique, the dose of heparin and the point of heparin addition to the assay are implicit in this lack of congruity, and the matter remains unresolved. A further complication has arisen over the interaction of heparin and EDTA together in the insulin radioimmunoassay (Grant, 1972; Orosz, 1973), but heparin-plasma supplemented with EDTA is still believed to provide the most reliable estimates of immunoreactive insulin in blood (Orosz, 1973).

Recent studies have shown that an under-estimation of insulin occurs in haemolysed plasma samples (Brodal, 1971a; Cantrell, Hochholzer & Frings, 1972). This is probably due to the action of a glutathione-dependent enzyme present in the red cells (and haemolysed plasma) which degrades insulin into its component A & B chains (Brodal, 1971b). In the present studies plasma samples with any visible haemolysis were not accepted for assay.

As previously noted, the interpretation of IRI values for rat insulin reported here is complicated by the "relative" nature of the assay, which provides an estimate of rat insulin in terms of human insulin equivalents. A further difficulty of interpretation arises from the identification of two types of insulin and proinsulin in the rat (Clark & Steiner, 1969; Smith, 1972). The amino acid sequences of the two molecules differ from each other at residues B9 and B29 (fig.4) and each is derived from a separate proinsulin precursor. The proinsulin fraction may not be distinguished immunologically from the insulin fraction, and some proinsulin appears to be secreted by the pancreatic islets together with the insulin and c-peptide (Clark & Steiner, 1969).

It has been assumed that both types of rat insulin occur in equal proportions in blood (Taylor, Howell, Montague & Edwards, 1968). Using bovine insulin antibody this group has compared the standard curves of bovine insulin and rat insulins (in equal proportions) by the Hales & Randle (1963a) double antibody method. Considerable cross-reactivity between the bovine insulin antibody and the rat insulins was observed, and with the particular batch of antisera tested, the rat insulins tended to be underestimated, especially at higher concentrations. O'Connor (1973), using the same assay system, compared the standard curves of bovine insulin and rat insulins (in equal proportions) against several different batches of bovine insulin antibody. The extent of cross-reactivity varies with each batch of antisera and some antisera tended to over-estimate the rat insulins.

When the present studies were begun highly purified rat

insulins were not available and comparisons of human and rat insulin standards in the present assay system have not been made. Since the extent to which rat insulin cross-reacts with each batch of antisera is likely to vary, the same batch of antisera has been used in all experiments in which IRI values are directly compared.

Glossary

The following definitions have been accepted in this chapter:

"Absolute" assay: standard and unknown hormone react identically with the antibody and provide a true measure of the concentration of the unknown hormone (Berson & Yalow, 1968).

Accuracy: the extent to which the mean of an infinite number of measurements of a hormone agrees with the exact amount of the hormone which is present (Midgley, Niswender & Regar, 1969).

Affinity: the energy of binding of an antigen for a particular antibody (Hurn & Landon, 1971).

Avidity: the energy of binding of an antibody for a particular antigen (Hurn & Landon, 1971).

Complement: factors present in the non-cellular fraction of blood which, when activated, may diminish the strength of the antigen-antibody reaction (Skelley et al., 1973).

Precision: the extent to which a given set of measurements of the same sample agrees with the mean, i.e. the amount of variation in the estimation of unlabelled hormone (Midgley et al., 1969).

"Relative" assay: standard and unknown hormone react differently with the antibody and provide an estimate of the concentration of the unknown hormone which is relative to the standard hormone (Berson & Yalow, 1968).

Reproducibility: the extent to which an estimate is duplicated upon repeat measurement (Midgley et al., 1969).

Specificity: the extent of freedom from interference by substances other than the one intended to be measured (Midgley et al., 1969).

Sensitivity: the smallest amount of unlabelled hormone which may be distinguished from no hormone (Midgley et al., 1969).

Table 3. METHODS USED TO SEPARATE ANTIBODY-BOUND
FROM FREE LABELLED INSULIN*

a. Differential migration, by chromatoelectrophoresis gel filtration (Sephadex G75) wick-chromatography	Yalow & Berson, 1960 Genuth et al. 1965 Orskov, 1967
b. Precipitation of antibody-bound insulin with salt (sodium sulphite) a secondary antibody " " " " " " alcohol (80% ethanol)	Grodsky & Forsham, 1960 Hales & Randle, 1963a, b Morgan & Lazarow, 1963 Goetz et al. 1963 Heding, 1966
c. Adsorption of free labelled insulin, on to ion exchange resin (Amberlite CG-400) dextran-coated charcoal finely divided silica or talc cellulose powder uncoated charcoal	Meade & Klitgaard, 1962 Herbert et al. 1965 Rosselin et al. 1966 Zaharko & Beck, 1968 Albano & Ekins, 1970
d. Enzyme proteolysis of free labelled insulin, using insulinase glutathione-activated ficin	Beck et al. 1964 Mitchell & Byron, 1967

* Only original descriptions of the various separation
methods are cited in this table

Figure 4

The amino acid sequences of rat, human, bovine, and porcine insulins.

<u>Residue</u>	<u>A Chain</u>			
	<u>Rat</u> (types 1 & 2)	<u>Human</u>	<u>Bovine</u>	<u>Porcine</u>
1	Gly			
2	Ile			
3	Val			
4	Asp	Glu	Glu	Glu
5	Gln			
6	Cys			
7	Cys			
8	Thr		Ala	
9	Ser			
10	Ile		Val	
11	Cys			
12	Ser			
13	Leu			
14	Tyr			
15	Gln			
16	Leu			
17	Glu			
18	Asn			
19	Asn			
20	Cys			
21	Asn			

Figure 4 (cont.)

<u>Residue</u>	<u>Rat 1</u>	<u>B Chain</u>			
		<u>Rat 2</u>	<u>Human</u>	<u>Bovine</u>	<u>Porcine</u>
1	Phe				
2	Val				
3	Lys				
4	Glu				
5	His				
6	Leu				
7	Cys				
8	Gly				
9	Pro	Ser	Ser	Ser	Ser
10	His				
11	Leu				
12	Val				
13	Glu				
14	Ala				
15	Leu				
16	Tyr				
17	Leu				
18	Val				
19	Cys				
20	Gly				
21	Glu				
22	Arg				
23	Gly				
24	Phe				
25	Phe				
26	Tyr				
27	Thr				
28	Pro				
29	Lys	Met	Lys	Lys	Lys
30	Ser	Ser	Thr	Ala	Ala

Adapted from Smith, (1972).

Figure 5.

Flow diagram to illustrate the essential features of the insulin radioimmunoassay used in the present study.

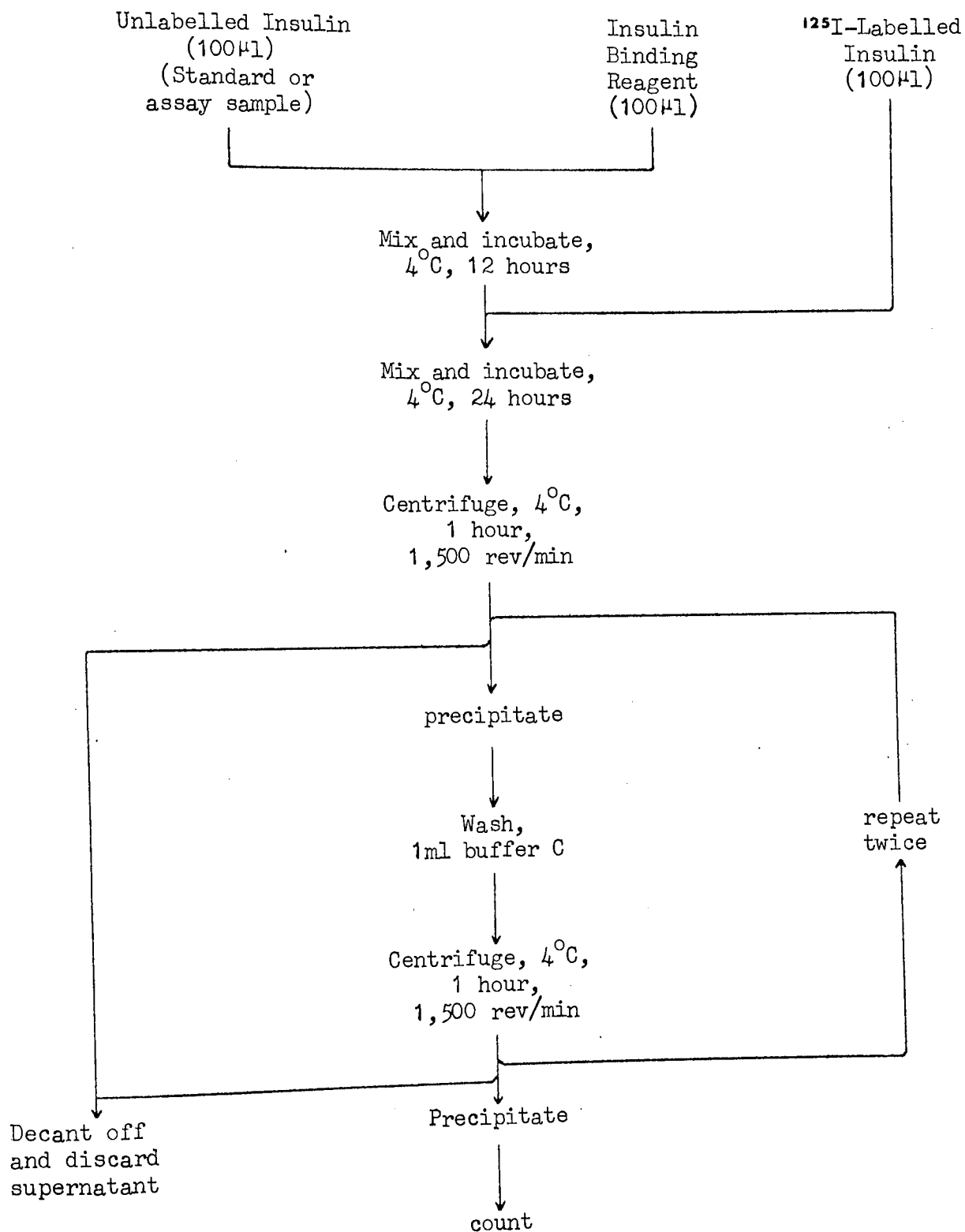


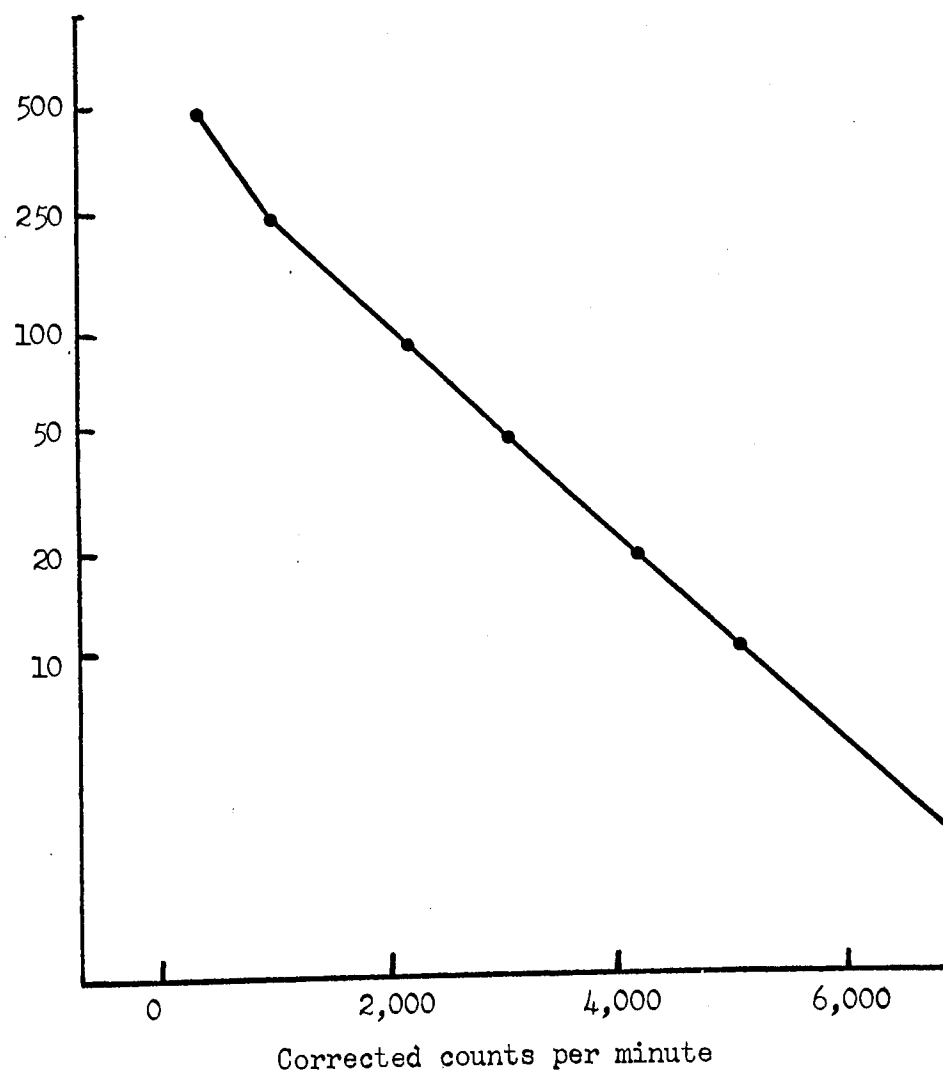
Figure 6

Program constructed for use with the Olivetti
Programma 101 desk computer to correct and average
where necessary counts obtained in the insulin
radioimmunoassay.

	Instruction typed-in	Computer Print-out
	Record Program	
Begin program	AV	AV
	S	MS
	A↓	M↓
enter replicates into (S	MS
arithmetic store and (+	M+
add replicates (S	MS
	+	M+
divide by (S	MS
number of replicates (÷	M÷
subtract quantity in register B	B-	B-
divide by quantity in register C	C÷	C÷
print out result	A◇	A◇
space at end of program	/◇	/◇
return to beginning of program	V	MV
Stop recording program		
enter (background 200	200 B↑	200 B↑
constants (decay factor 1.346	1.3460 C↑	1.3460 C↑
Begin operation of program	V	V
enter replicate counts,		
e.g. 5106	5106 S	5106 S
4981	4981 S	4981 S
5009	5009 S	5009 S
enter number of replicates	3 S	3 S
Result (corrected average count)		3515 A◇
prints out		

Figure 7 Typical standard curve for insulin radioimmunoassay

Insulin concentration
(μ U/ml)
plotted on a
logarithmic scale



CHAPTER 4

OESTROUS CYCLE AND ONE WEEK
OVARIECTOMY IN FEMALE RATS

OESTROUS CYCLE AND ONE WEEK OVARECTOMY
IN FEMALE RATS

Introduction

The oestrous cycle is associated with rhythmical fluctuations of many biological parameters. Cyclical changes of the structure and functions of the ovaries and reproductive tracts are well documented (Long & Evans, 1922; Eckstein, 1962; Eckstein & Zuckerman, 1965a) and behavioural and other features of the cycles have been reviewed by Eckstein & Zuckerman (1965b).

Recently, variations in the circulating levels of sex hormones during the cycle have been quantified. The circulating levels of oestrogens (Yoshinaga et al., 1969; Shaikh, 1971), progestins (Feder, Resko & Goy, 1968; Hashimoto et al., 1968; Uchida, Kadowaki & Miyake, 1969) and luteinizing hormone (Monroe, Rebar, Gay & Midgley, 1969; Daane & Parlow, 1970) rise rapidly during pro-oestrus and reach their respective peaks between 14.00 and 17.00 hours. The level of follicle stimulating hormone rises less rapidly and does not become maximal until early oestrus (Daane & Parlow, 1971). During oestrus the circulating levels of sex hormones decline, and with the exception of a secondary peak of progestins at mid-metoeestrus, the levels during the other stages of the cycle are minimal. Current knowledge of the factors that regulate sex hormone secretion during the cycle has been reviewed by Schwartz (1969) and Schally, Arimura & Kastin (1973).

Following ovariectomy the circulating levels of sex steroids

generally fall below the normal dioestrus values, but detectable levels are maintained through the continued secretion of sex steroids by the adrenal glands (Feder, Resko & Goy, 1968; Labhsetivar, 1972; Saez, Morera, Dazord & Bertrand, 1972). In contrast, however, the amount of circulating follicle stimulating hormone rises markedly and the circulating level of luteinizing hormone becomes considerably elevated above the dioestrus value (Gay & Midgley, 1969; Swerdloff, Jacobs & Odell, 1972).

The experiments described in this chapter were undertaken to investigate whether there are changes of glucose tolerance and plasma insulin during the oestrous cycle and one week following ovariectomy in the female rat.

Materials and methods

The oestrous cycle study was conducted using adult virgin female rats of 12 weeks of age and weighing about 230g. The animals were housed in groups of 6 per cage and the lighting schedule was adjusted to 12 hours light (09.00 to 21.00 hours) and 12 hours darkness to facilitate the synchrony and regularisation of the cycles. Vaginal smears were examined daily at approximately 12.00 hours and only animals that had shown four consecutive regular 4-day cycles were accepted for experimentation.

A similar group of animals were bilaterally ovariectomized at 11 weeks of age and experiments were performed one week later.

Intraperitoneal glucose tolerance and plasma insulin levels were determined on each of the days of the oestrous cycle and one week following ovariectomy. Each animal was used for only one measurement.

Results

During the oestrous cycle compatible changes of glucose tolerance and plasma insulin were observed (figs 8 and 9). Glucose tolerance was greatest and plasma insulin levels were highest on the day of oestrus. There was a reduction of both of these parameters at metoestrus and this trend was continued into dioestrus. At pro-oestrus glucose tolerance was improved and plasma insulin levels were elevated almost to the oestrus values.

Statistical comparisons of the mean ipGT_A for each stage of the cycle revealed that glucose tolerance was significantly impaired at dioestrus with respect to the other three stages (pro-oestrus versus dioestrus, $p < 0.001$; oestrus versus dioestrus, $p < 0.001$; metoestrus versus dioestrus, $p < 0.02$). Differences between the other stages were not significant. Statistical comparisons of the mean plasma insulin levels showed that the level at dioestrus was significantly lower than that at oestrus ($p < 0.05$) but there were no significant differences between the levels recorded at the other stages.

Glucose tolerance and plasma insulin levels in the ovariectomized animals were very similar to those of the intact dioestrus individuals.

Discussion

These changes of glucose tolerance and plasma insulin levels during the oestrous cycle and following ovariectomy may be related to the endogenous concentrations of circulating sex hormones. The relatively greater glucose tolerance and higher

plasma insulin levels at pro-oestrus and oestrus might be associated with the rise of circulating sex hormones during pro-oestrus. The fact that glucose tolerance and plasma insulin levels were maximal at oestrus, when sex hormone titers have declined, might reflect the timing of the experiments with respect to the time-course of action of the hormones.

Physiological changes induced by oestrogens, for example, may not become manifest for several hours (Jensen & Desombre, 1972), thus the elevated sex hormone levels at pro-oestrus might exert a greater influence on carbohydrate metabolism during oestrus than during pro-oestrus. Similarly, a secondary peak of progestins during metoestrus might be associated with the intermediary values of glucose tolerance and plasma insulin at this stage, while the paucity of circulating sex hormones at dioestrus corresponds to reduced glucose tolerance and lowered plasma insulin levels. Furthermore, the glucose tolerance and plasma insulin values of ovariectomized animals closely resemble those of intact dioestrus individuals; and this may be equated with the net influence of reduced sex steroid levels and increased gonadotrophin levels in the blood of ovariectomized animals in comparison with the dioestrus condition.

The oestrus cycle is accompanied by cyclical alterations of other endocrine phenomena that might influence carbohydrate metabolism. The secretion of corticosterone and aldosterone follows a similar cyclical pattern to that of the sex steroids (Dean, Cole & Chester Jones, 1959; Hinsull & Crocker, 1970; Raps, Barthe & Desaulles, 1970) while melatonin synthesis is inversely related to the sex steroid levels (Wurtman, Axelrod,

Synder & Chu, 1965). The possible involvement of these hormones will be considered in later chapters.

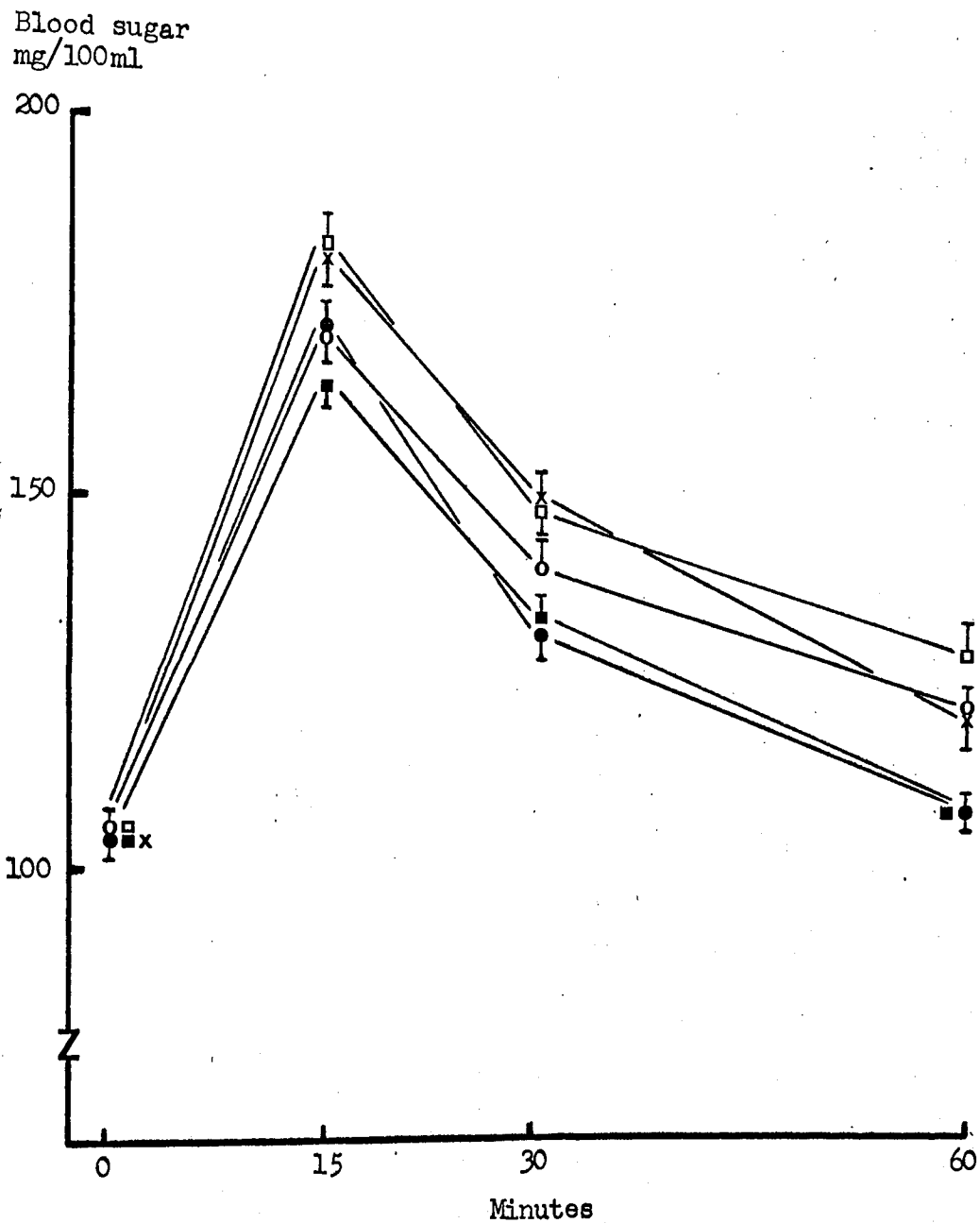
It is likely that the feeding and activity patterns of the animals bear an important influence on the changes of glucose tolerance and plasma insulin observed. There is a decrease of food consumption and an increase of voluntary activity at around the time of oestrus, and following ovariectomy the consumption of food may exceed that of the dioestrus animal, while voluntary activity becomes considerably reduced (Slonaker, 1924, 1925; Wang, 1923; Tartellin & Gorski, 1971; Paul & Dutttagupta, 1973). Extensive evidence has been advanced to relate these changes with the levels of gonadal steroids (see review by Wade, 1972).

In accordance with the present study, Paul & Dutttagupta (1973) have reported that the glycogen content of rat liver and uterus is increased at oestrus, and following ovariectomy the glycogen content of each tissue declines to slightly below the dioestrus level. These authors have implicated the involvement of sex steroids.

The lack of conclusive evidence for a rhythmical fluctuation of carbohydrate metabolism during the menstrual cycle of healthy women was mentioned in chapter one. However, the present study is consistent with observations of a deterioration in the condition of women diabetics during the menses (Leading article, 1970), when the circulating levels of sex hormones are reduced.

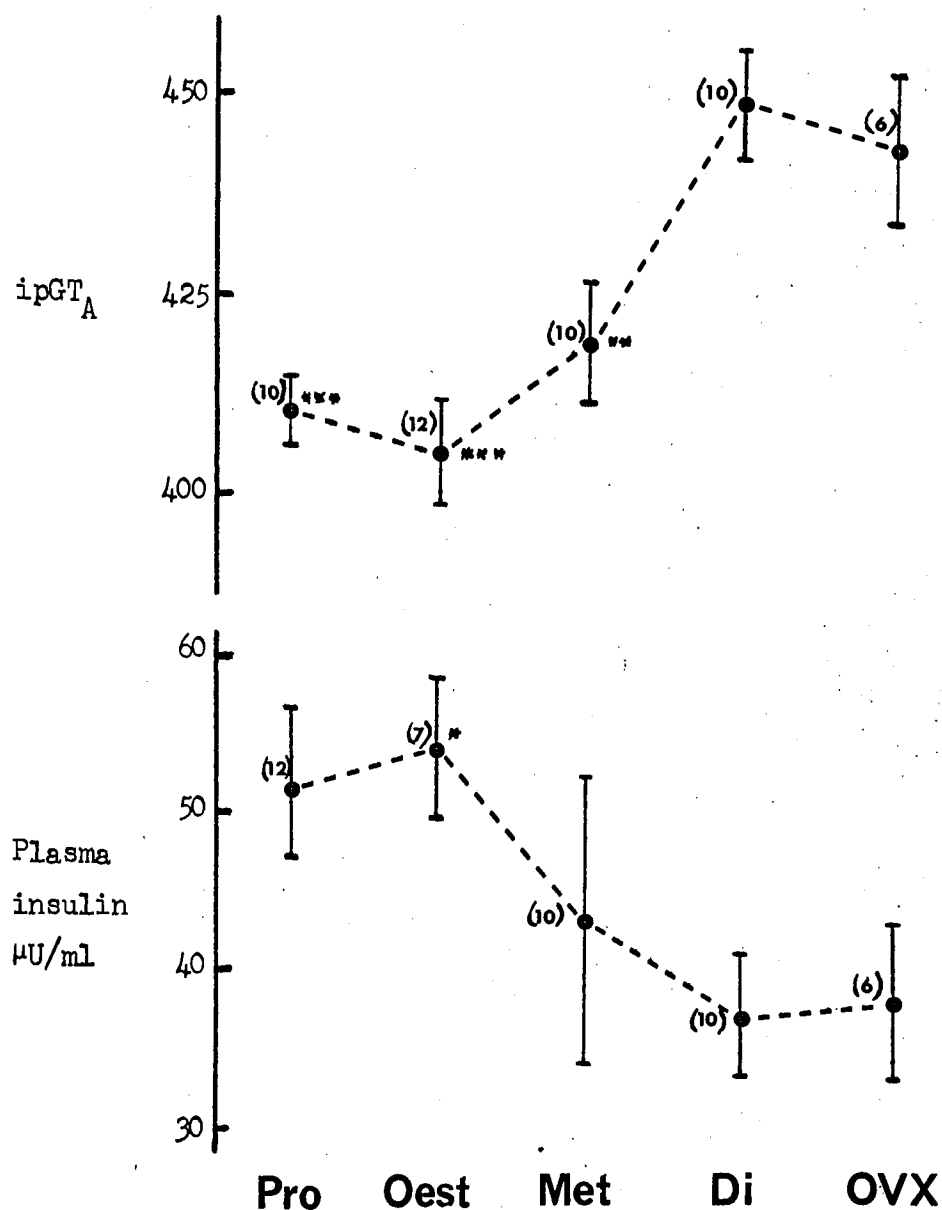
Thus, it is tentatively considered that higher endogenous sex hormone titers may be correlated with improved glucose tolerance and elevated plasma insulin levels.

Figure 8 Intraperitoneal glucose tolerance during the oestrous cycle and one week ovariectomy (mean \pm sem)



- — ● pro-oestrus (n = 10)
- — ■ oestrus (n = 12)
- — ○ metoestrus (n = 10)
- — □ dioestrus (n = 10)
- x — x one week ovariectomy (n = 6)

Figure 9 Intraperitoneal glucose tolerance area and basal plasma insulin level during the oestrous cycle and one week after ovariectomy (mean \pm sem)



Pro - pro-oestrus
 Oest - oestrus
 Met - metoestrus
 Di - dioestrus
 OVX - one week ovariectomy

*p < 0.05; ** p < 0.02; *** p < 0.001; all values compared with dioestrus

Number of observations in parentheses

CHAPTER 5

AGE AND LONG TERM OVARECTOMY
IN FEMALE RATS

AGE AND LONG TERM OVARECTOMY
IN FEMALE RATS

Introduction

Although it is generally agreed that carbohydrate tolerance deteriorates throughout adult life, the mechanisms underlying this process remain unestablished. Age-related alterations of insulin metabolism have been the subject of several studies, but conclusive data have yet to be obtained. It is possible that age-related modifications of other components of the endocrine environment are contributing to the reduction of carbohydrate tolerance, and Zeytinoglu & Gherondache (1970) have particularly implicated the hormonal changes that follow the menopause in women. These changes include striking alterations of the circulating levels of sex hormones: the circulating levels of gonadal steroids decline rapidly while those of pituitary gonadotrophins become raised for about two decades before they too decline (McGavack, 1967; Bertolini, 1969; Papanicolaou, Loraine & Dove, 1969; Dove, Morley, Batchelo & Lunn, 1971).

A cessation of reproductive potential equivalent to the human menopause does not take place in the female rat. Although there have been no reports directly concerned with the levels of sex hormones in the aged female rat, it is known that reproductive capacity becomes reduced, and Peng & Huang (1972) have suggested that this may reflect a decline of hypothalamic-pituitary-ovarian endocrine function.

The hormonal status of post-menopausal women is very similar to that induced by ovariectomy, and the experiments described in the present chapter were designed to investigate the influence of this endocrine environment on carbohydrate metabolism, with particular reference to the ageing female. To this effect, the changes of glucose tolerance and circulating insulin that occur in the intact aged female rat have been compared with those that occur in aged individuals after long term ovariectomy.

Materials and methods

Intraperitoneal glucose tolerance and plasma insulin levels were determined at 40 weeks of age in normal intact female rats in the reproductive state of dioestrus, and in individuals that had been ovariectomized at 11 weeks of age. The experimental procedures were identical to those described in the previous chapter; thus the two sets of data may be directly compared.

Similar groups of 40 week old intact dioestrus and ovariectomized animals were studied using intravenous glucose tolerance tests and a histological survey of the pancreatic islets was also performed. Histological sections were derived from the splenic portion of pancreata removed at 15.00 hr. from animals previously fasted for 6 hours and killed by a blow on the head. The sections were stained with either H.E., Gomori's A.F., or the silver impregnation technique of Grimelius.

Results

The intraperitoneal glucose tolerance curves in young (12 week) and old (40 week) intact dioestrus and ovariectomized female rats are illustrated in figure 10. Both intact and

ovariectomized animals showed an impairment of glucose tolerance with age, although this was statistically significant only for the ovariectomized animals (young ovariectomized versus old ovariectomized, $p < 0.01$). The glucose tolerance curve of the old ovariectomized group was also significantly impaired with respect to the intact group of the same age ($p < 0.05$).

The basal blood sugar and plasma insulin levels determined at 15.00 hr in the 6 hour fasted animals did not show any significant differences between the four groups (table 4).

The arterial plasma insulin and blood sugar levels determined during ivGTTs in the 40 week intact and ovariectomized animals are illustrated in figure 11. No statistically significant differences between the two groups were observed.

The fasting (18 hours, overnight) blood sugar and plasma insulin levels determined at the start of these tests, together with values determined in 12 week intact dioestrus females under identical conditions are shown in table 4. (Levels recorded at other times during ivGTTs in the 12 week animals are not directly comparable with those determined in the old intact and ovariectomized groups since a steroid vehicle solution was injected with the glucose). The fasting values of both blood sugar and plasma insulin showed a tendency to increase with age and with ovariectomy, but not to the extent of statistical significance.

The histological examination of the pancreatic islets in the 40 week intact and ovariectomized animals did not reveal any gross structural differences between the two groups. Normal quantities of aldehyde fuchsin-positive staining granular material and a

normal distribution of argyrophilic-positive staining tissue were observed (plate A).

The 40 week ovariectomized animals were considerably heavier than their intact counterparts and appeared obese.

Discussion

Intact dioestrus females

The age-related changes of glucose tolerance and plasma insulin observed in the present investigation are substantially consistent with previous studies in the rat. These studies have indicated a tendency for the fasting blood sugar and plasma insulin levels to become raised in the older animals, but there is a lack of agreement on the extent of this rise. A slight, though non-significant elevation of the fasting blood sugar and plasma insulin values was observed in the present study (over the age range of 12 to 40 weeks) but a similar trend was not apparent in the non-fasting state. It is suggested that the incomplete congruity among previous studies may reflect differences in the age, sex or period of fasting of the animals, and in the source of the blood sample (arterial or venous) and the time of day at which it is obtained.

In agreement with other reports there was a tendency for glucose tolerance to deteriorate with advancing age, but this was not significant over the age range studied.

The present study did not reveal a consistent alteration of the basal plasma insulin level in the older age group. The recent work of Gommers & De Gasparo (1972) has provided evidence in the rat to support the theory of Marigo (1968) that the age-

related changes of glucose metabolism are accompanied by an initial phase of hyperinsulinism and a subsequent phase of hypoinsulinism. Data obtained in the present study, however, do not include a sufficiently extended age range to be considered in the context of this theory.

Ovariectomized females

The deterioration of glucose tolerance in the ageing female rat was accentuated by long term ovariectomy. The insulin response to a glucose challenge was not significantly altered by ovariectomy but evidence of a slightly enhanced response was obtained. Furthermore, in both the fasting and the non-fasting ovariectomized animals the basal insulin levels tended to be raised. The impairment of glucose utilization observed during ipGTTs was not reflected in the arterial blood sugar levels following a glucose challenge.

Impaired glucose utilization accompanied by unimpaired or enhanced insulin secretion may be correlated with the obese constitution of the ovariectomized animals. These features may be attributable to increased insulin resistance associated with a slight compensatory hyper-insulin secretion (see Rabinowitz, 1970). A relationship between obesity and maturity-onset diabetes under conditions of compromised insulin reserve has been shown in several studies (see Stern & Hirsch, 1972).

Thus it appears that ovariectomy advances the normal age-related deterioration of glucose metabolism in the female rat. This is consistent with the view that reduced gonadal endocrine function in the ageing female contributes towards an impairment of glucose metabolism, which may become manifest in the

form of obesity and/or diabetes, depending on the adaptive insulin secretory capacity of the islets.

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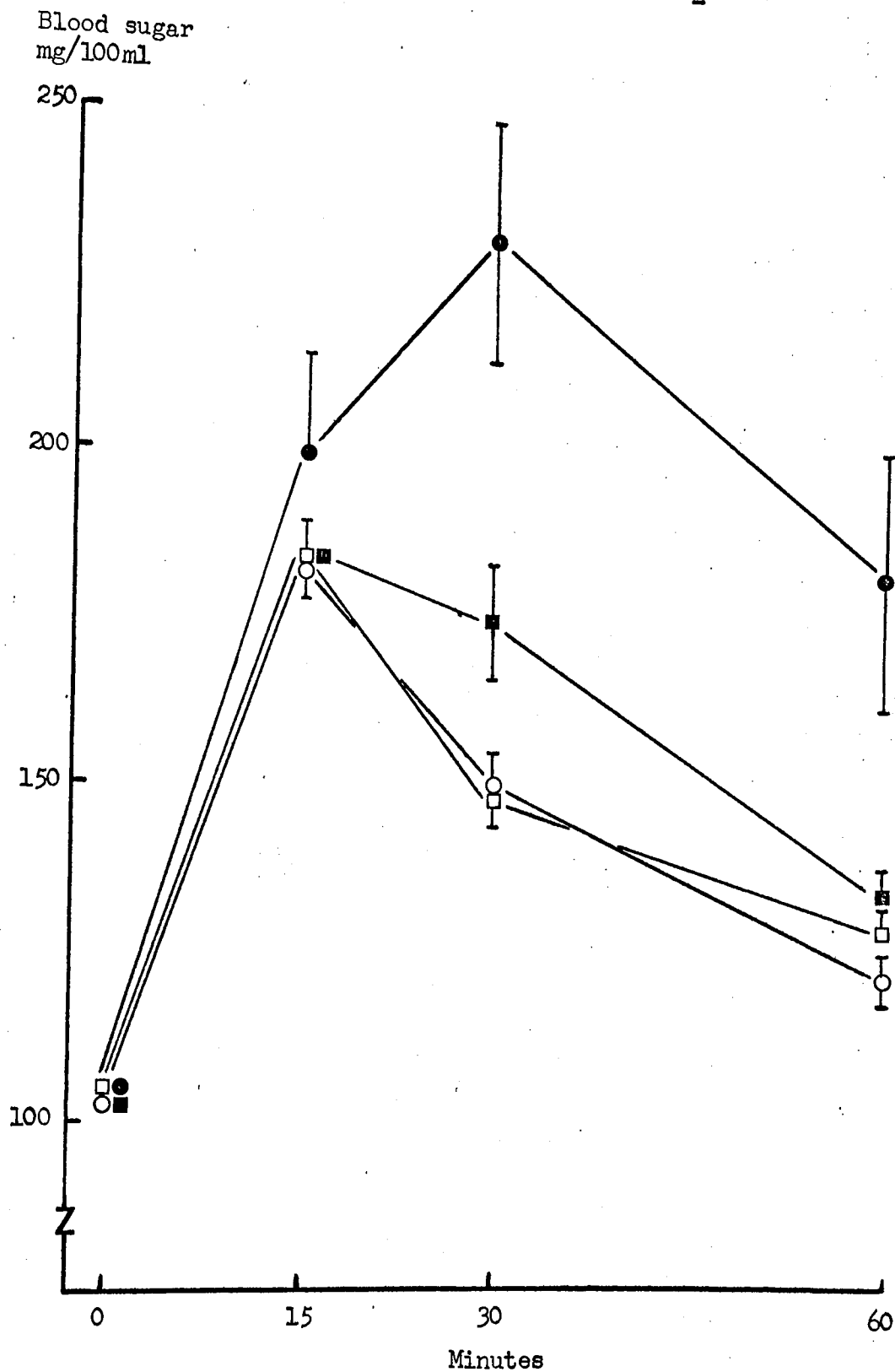
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TABLE 4. BLOOD SUGAR AND PLASMA INSULIN LEVEL IN YOUNG (12 week) and OLD (40 week)

INTACT DIOESTRUS AND OVARIECTOMIZED FEMALE RATS

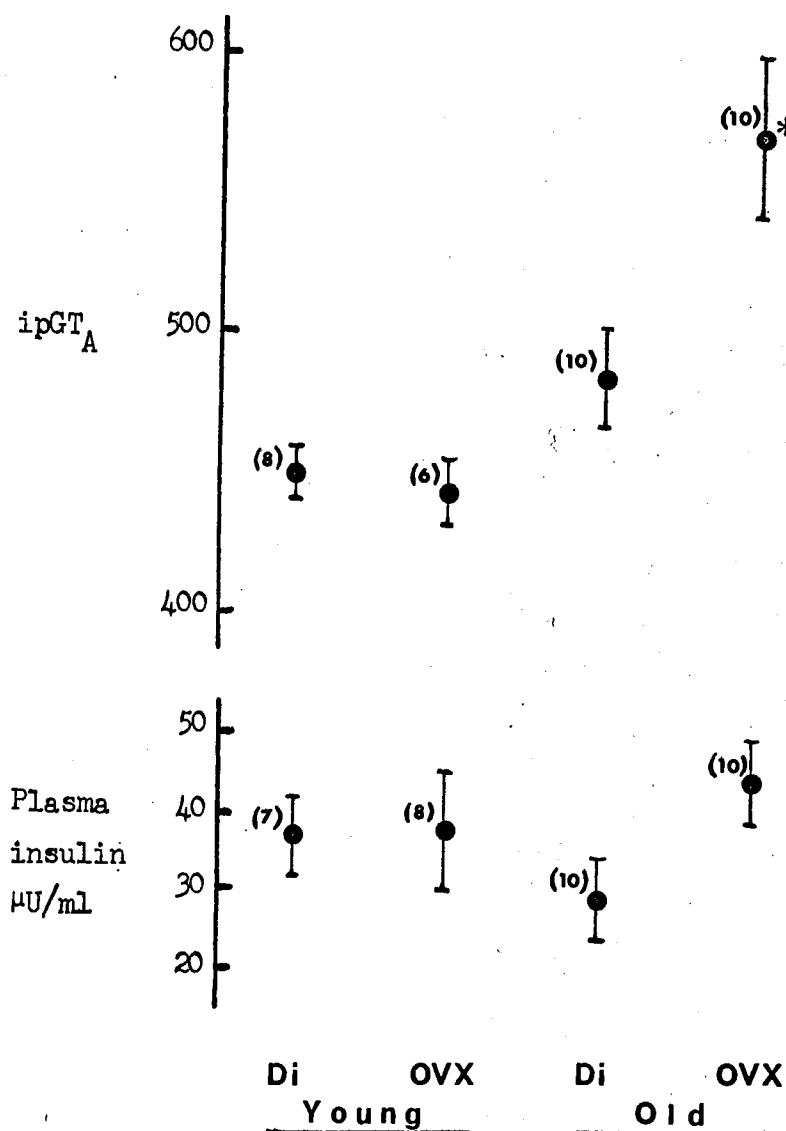
		12 weeks of age		40 weeks of age	
		Intact Dioestrus	Ovari- ectomized	Intact Dioestrus	Ovariectomized
6 HOUR	Blood Sugar (mg/100 ml)	106.4 \pm 1.9	104.6 \pm 2.4	103.5 \pm 1.7	105.1 \pm 1.3
FAST	Plasma Insulin (μ U/ml)	37.4 \pm 4.3	38.2 \pm 5.8	35.0 \pm 3.9	44.5 \pm 3.8
18 HOUR	Blood Sugar (mg/100 ml)	95.3 \pm 2.6	-	102.0 \pm 2.1	109.0 \pm 4.2
FAST	Plasma Insulin (μ U/ml)	17.5 \pm 2.5	-	24.5 \pm 2.9	31.0 \pm 4.3

Figure 10a Intraperitoneal glucose tolerance in young (12 week) and old (40 week) intact dioestrus and ovariectomized female rats (mean \pm sem)



- young intact dioestrus (n = 10)
- young ovariectomized (n = 6)
- old intact dioestrus (n = 8)
- old ovariectomized (n = 7)

Figure 10b Intraperitoneal glucose tolerance area and basal plasma insulin level in young (12 week) and old (40 week) intact dioestrus and ovariectomized female rats (mean \pm sem)



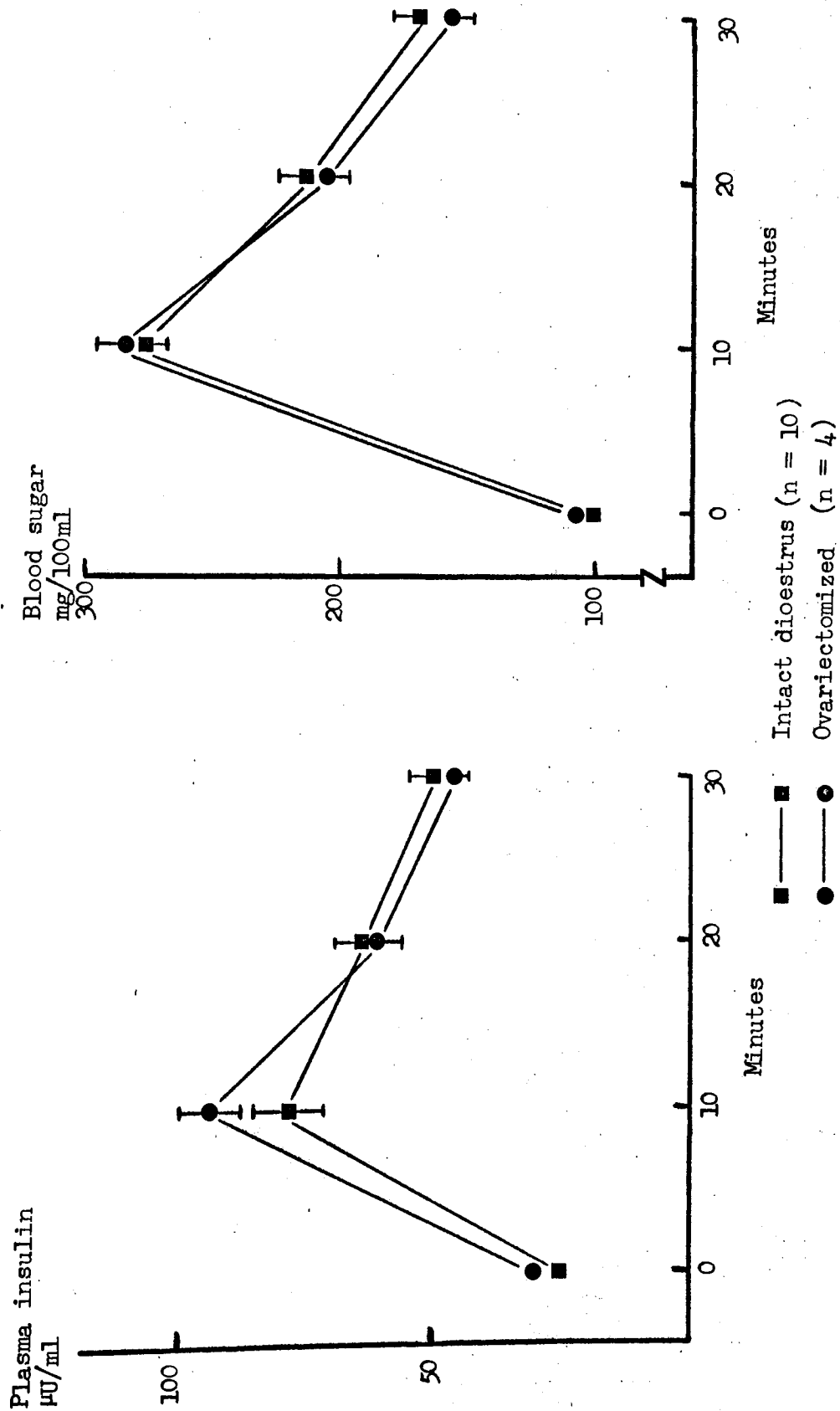
Di - intact dioestrus

OVX - ovariectomized

* $p < 0.01$ compared with young ovariectomized rats; $p < 0.05$ compared with old intact dioestrus rats

Number of observations in parentheses

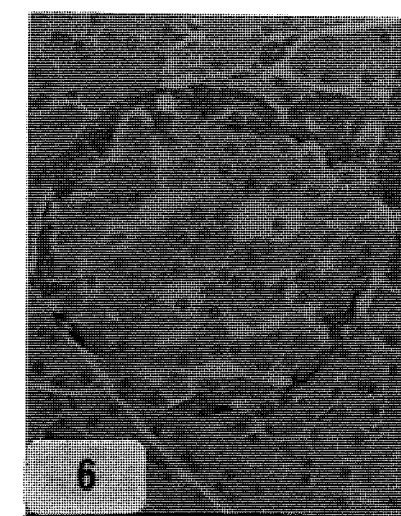
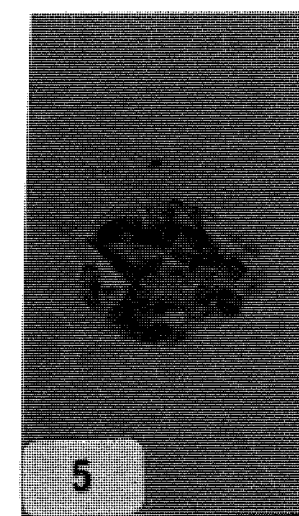
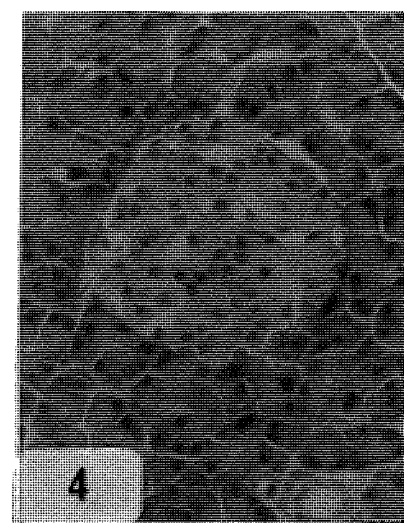
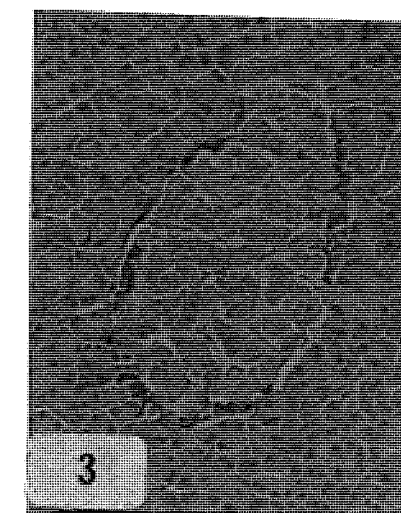
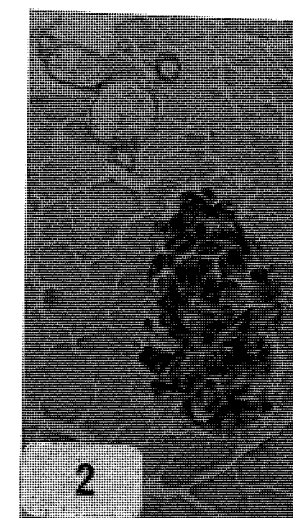
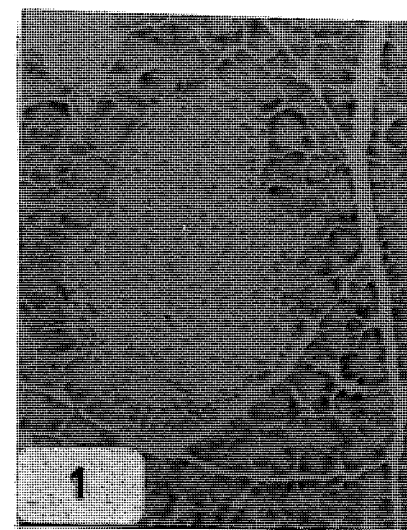
Figure 11 Intravenous glucose tolerance in old (40 week) intact dioestrus and ovariectomized female rats (mean \pm sem)



Description of Plate A

Islets of Langerhans of old (40 week) intact dioestrus and ovariectomized female rats

- 1 Islet from intact dioestrus individual stained with H.E. (x 50).
- 2 Islet from intact dioestrus individual stained with A.F. (x 50).
- 3 Islet from intact dioestrus individual stained with the silver impregnation technique of Grimelius (x 50).
- 4 Islet from ovariectomized individual stained with H.E. (x 80).
- 5 Islet from ovariectomized individual stained with A.F. (x 80).
- 6 Islet from ovariectomized individual stained with the silver impregnation technique of Grimelius (x 80).



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CHAPTER 6

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EFFECTS OF SEX HORMONES IN
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EFFECTS OF SEX HORMONES IN OVARECTOMIZED FEMALE RATS

Introduction

The changes of glucose homeostasis observed during the oestrous cycle and following ovariectomy in the female rat indicate that sex hormones may be contributing to the regulation of carbohydrate metabolism. Since there is considerable circumstantial evidence in the literature to support this view, the present study was undertaken to examine the effects of individual sex hormones on glucose tolerance and plasma insulin levels in ovariectomized female rats.

Materials and methods

Adult virgin female rats were ovariectomized at 10 weeks of age and hormone treatments were commenced two weeks post-operatively. Weight matched groups of animals (approximately 200g) were given daily intramuscular injections at 10.00 hr for 14 days: oestradiol benzoate, 5 µg; progesterone, 5 µg; human menopausal gonadotrophin (HMG), 5 i u of FSH and 5 i u of LH, and methyl testosterone, 5 µg. Control animals were ovariectomized and injected with vehicle only, 0.05 ml of arachis oil. On the 14th day intraperitoneal glucose tolerance or basal plasma insulin levels were determined as previously described. The insulin values reported in this chapter may not be directly compared with those reported in the previous chapters because a different batch of insulin antisera was employed in the radioimmunoassay.

Similar doses (by weight) of oestradiol, progesterone and testosterone were employed in this study to obtain information on the relative potencies of these hormones on the parameters measured. The dose selected, $2.5 \mu\text{g}/100\text{g}$ body weight was chosen to compromise the replacement doses that have been customarily assumed by other workers (Zarrow et al., 1964). Treatment with $0.1 \mu\text{g}/100\text{g}$ body weight/day of oestradiol is considered to provide a physiological replacement of oestrogen in the ovariectomized female rat, whereas a considerably larger dose of progesterone is recommended since this hormone appears to be more rapidly metabolized. Testosterone replacement in castrated male rats has been variously estimated, but the dose used in this study appears to be physiologically acceptable in that sex. The dose of HMG administered corresponds (on a weight for weight basis) to the maximum that is advised for the treatment of anovulation in amenorrhoeic women (Searle Scientific Services, 1969). This is greatly in excess of the physiological range.

The hormones were administered intramuscularly to prevent rapid entry into the circulation and therapy was extended over 14 days to allow adequate time for the animals to readjust to the new endocrine environment. It is appreciated that the steroid treatments may completely saturate the tissue receptor mechanisms and that smaller doses may be equally effective. However, available evidence suggests that this may only appertain to the accumulation of steroids in "target" tissues (James & Fotherby, 1970).

Results

The glucose curves observed in the control and hormone treated groups of animals are illustrated in figure 12. Glucose tolerance was improved in the animals receiving oestradiol, progesterone or HMG, but in the animals receiving testosterone glucose tolerance was impaired. Statistical evaluation of the $ipGT_A$ for each group (fig.13) shows that these changes were statistically significant. The hormones that improved glucose tolerance also tended to lower the basal blood sugar level but this was only significant for the oestradiol treated group. Testosterone significantly elevated the basal blood sugar level.

Basal plasma insulin levels were significantly raised in the oestradiol, progesterone and HMG treated groups but consistent changes were not apparent in the group receiving testosterone (fig.13).

Discussion

The present data indicate that oestradiol and progesterone improve glucose tolerance and concomitantly raise plasma insulin levels in the female rat. Costrini & Kalkhoff (1971) have recently reported similar results following an almost identical series of experiments. It appears that at the doses employed, oestradiol and progesterone improve glucose tolerance to approximately the same extent although oestradiol exerts a considerably greater insulinotropic effect. These observations suggest a physiological role of oestradiol and progesterone in the regulation of carbohydrate metabolism but it seems unlikely that they operate through identical mechanisms.

HMG also produced an improvement of glucose tolerance and

an elevation of plasma insulin levels in the female rat but the physiological significance of these effects remains uncertain since the dose employed was in the supraphysiological range. The possibility exists that the HMG preparation (Pergonal) contains trace amounts of contaminant material that may be contributing to the observed effects. The distributors of the preparation (G. D. Searle & Company Ltd.) were unable to provide conclusive evidence to the contrary. It is not unreasonable to suppose that the effects produced by the HMG preparation legitimately belong to the FSH-LH mixture since a number of recent studies have suggested that pituitary gonadotrophins are not solely concerned with gonadal regulation. Cushman (1969) has demonstrated that HMG enhances glucocorticoid secretion, and there is evidence that LH provokes lipolysis of adipose tissue (Gospodarowicz, 1971a, b) possibly through the adenyl cyclase system (Birnbaumer & Rodbell, 1969). Furthermore, Melloni & Pecchiri (1957) have reported a mild growth promoting effect of FSH on the pancreatic islets. However, these observations are not sufficient to indicate the possible mechanisms through which gonadotrophins may produce the changes of carbohydrate metabolism observed in the present study.

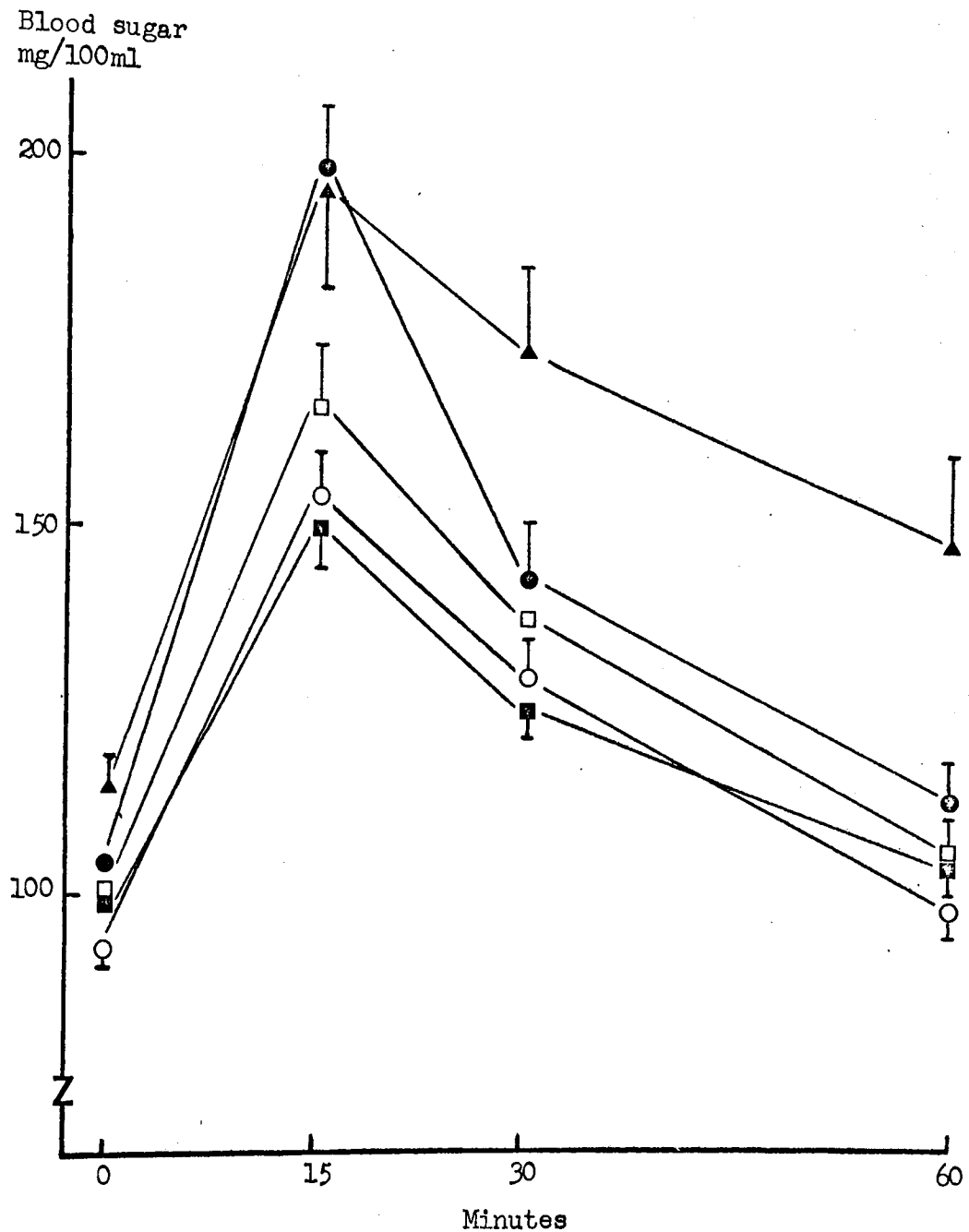
The effects of oestradiol, progesterone and gonadotrophin on glucose tolerance and plasma insulin levels in ovariectomized female rats, reported herein and by Costrini & Kalkhoff (1971) are compatible with the alterations of glucose tolerance and plasma insulin observed during the oestrous cycle and following ovariectomy. Support is thus given to the view that carbohydrate metabolism is influenced by the endogenous levels of sex hormones during different reproductive states in the female.

The present data are also in general agreement with previous studies in this area. Basabe et al. (1969) have observed a hyperinsulinaemic effect of oestradiol in female rats and Beck (1969b) has observed a similar effect in progesterone treated female monkeys. Hypoglycaemic tendencies of oestradiol and progesterone have been noted in several animal species (Beric & Karanfilski, 1957; Talaat et al., 1960; Benjamin & Casper, 1966; Planas et al., 1967; Basabe et al., 1969), and improved glucose tolerance and elevated plasma insulin levels have also been observed in women receiving preparations of these hormones (chapter 1).

The rise in basal blood sugar levels and the impairment of glucose tolerance observed in the animals receiving testosterone are not in accordance with previous studies, which have failed to identify any glycaemic alterations in testosterone treated females. Although variations of species, dosage and duration of treatment may contribute to this discrepancy, an entirely satisfactory explanation is not apparent. The effects of testosterone on carbohydrate metabolism are considered further in the following chapter since they appear to bear relevance to sexual differences in the glucose tolerance curve.

Mechanisms involved in the mediation of sex hormone induced changes of glucose tolerance and basal plasma insulin levels in ovariectomized female rats may not be inferred from the experiments described in this chapter; this aspect is considered in subsequent chapters.

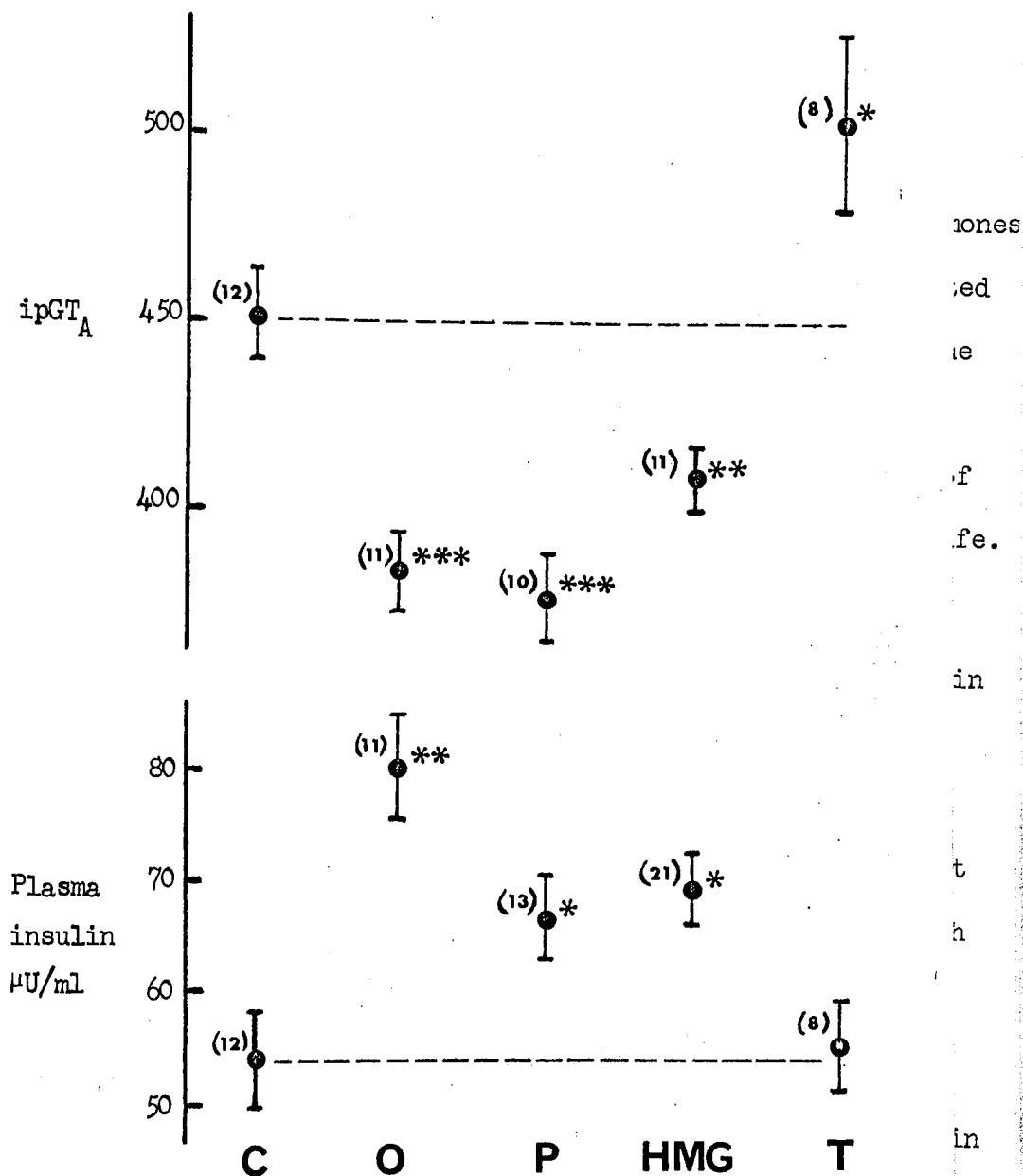
Figure 12 Intraperitoneal glucose tolerance in ovariectomized female rats treated with oestradiol, progesterone, HMG and testosterone (mean \pm sem)



- Control (n = 12)
- Oestradiol (n = 11)
- Progesterone (n = 10)
- HMG (n = 10)
- ▲—▲ Testosterone (n = 8)

All steroid treatments at dosage 2.5 μ g/100g body weight
HMG - 2.5iu FSH, 2.5iu LH/100g body weight

Figure 13 Intraperitoneal glucose tolerance area and basal plasma insulin level in ovariectomized female rats treated with oestradiol, progesterone, HMG and testosterone (mean \pm sem)



C - control
O - oestradiol
P - progesterone
HMG - gonadotrophin
T - testosterone

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; all values compared with control

Number of observations in parentheses

All steroid treatments at dosage

2.5 μ g/100g body weight

HMG - 2.5iu FSH, 2.5iu LH/100g body weight

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CHAPTER 7

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EFFECTS OF SEX HORMONES IN

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EFFECTS OF SEX HORMONES IN
ORCHIDECTOMIZED MALE RATS

Introduction

In contrast to the extensive literature relating sex hormones and carbohydrate metabolism in the female, there is only limited evidence to purport the existence of such a relationship in the male. This paucity of information may reflect the fact that males, unlike females, do not undergo any marked alterations of sexual endocrine function during the course of normal adult life.

It is generally held that testosterone promotes the deposition of glycogen into skeletal muscle, but it is uncertain whether glucose homeostasis is significantly altered. Talaat and co-workers (1957, 1964) reported that testosterone impairs glucose tolerance in rabbits, although a similar effect was not observed in man, and insulin insensitivity was detected in both studies.

The present investigation concerns the effects of sex hormones on glucose tolerance and basal plasma insulin levels in the orchidectomized male rat. The experimental design conforms to that described in the previous chapter; hence, the two sets of data provide comparative information on the effects of these hormones in each sex.

Materials and methods

Adult male rats were orchidectomized at 10 weeks of age and hormone treatments, identical to those described in the previous chapter were commenced two weeks post-operatively. The animals

used in this study weighed slightly more than their age-matched female counterparts. In addition to the hormone treated and control animals, a group of normal (intact and untreated) male rats of the same age and weight were also examined. Intra-peritoneal glucose tolerance and basal plasma insulin levels were determined as before.

Results

The glucose curves observed in the control and hormone treated groups of animals are illustrated in figure 14. There were no significant differences in the blood sugar levels recorded either before, or at the specified time intervals following the glucose challenge. Statistical evaluation of the $ipGT_A$ for each group (fig.15) also revealed the absence of any significant alterations. It is, however, worthy of note that there was a tendency towards impaired glucose tolerance in the animals receiving testosterone and progesterone since this was associated with a significant reduction of the basal plasma insulin levels (fig.15). Other hormone treatments did not produce any apparent alterations in plasma insulin levels.

A direct comparison of data obtained in the normal (intact and untreated) and control (orchidectomized and arachis oil treated) animals, in order to evaluate the effects of orchidectomy, may be criticised on the grounds that the latter (but not the former) received arachis oil injections. However, glucose tolerance and plasma insulin values observed in the two groups of animals were very similar.

Discussion

The observations recorded in the present and in the previous chapter indicate that sex hormones do not produce the same effects on glucose tolerance and plasma insulin levels in gonadectomized male and female rats.

Testosterone showed a tendency to reduce glucose tolerance in the male rat, which is not inconsistent with previous studies by Talaat et al. (1957, 1964) who observed a similar tendency in rabbits, but not in man. This condition is generally associated with a compensatory elevation of insulin secretion in healthy individuals with an adequate insulin reserve. Such an occurrence is not borne out by the reduced plasma insulin levels observed in the present study. The present data are considered to support the view that testosterone may influence the regulation of carbohydrate metabolism in the male, although it is unlikely that this hormone induces marked alterations in the glycaemic state of the animal.

The effects of testosterone observed in the male rats are in contrast to the marked impairment of glucose tolerance and apparently unaltered insulin levels observed in testosterone treated females. The absence of any consistent effects of oestradiol or HMG in the male are also in contrast to the improvement of glucose tolerance and the elevation of plasma insulin levels induced by these hormones in the female. The inactivity of oestradiol in the male is at further variance with a report by Talaat et al. (1965) that this hormone exerts a hyperglycaemic influence in castrated male rabbits. Progesterone, which exhibited hypoglycaemic and hyperinsulinaemic effects in

the female, showed the reverse tendencies in the male. These findings are not in line with those of Kalkhoff et al. (1970) who reported that progesterone raises plasma insulin levels without altering glucose tolerance in both men and women.

In seeking an explanation of the different effects of sex hormones on glucose tolerance and plasma insulin levels in gonadectomized male and female rats, the data so far accumulated were examined for possible inherent sex differences in carbohydrate metabolism: several interesting points emerged.

Sex differences

Apparent sex differences in certain aspects of carbohydrate metabolism have been noted in passing by a number of authors, but surprisingly little attention has been directed towards an understanding of this area. Moreover, the available evidence is not in general agreement and the nature of any sex differences thus remain in doubt.

The control groups of male and female rats (gonadectomized and arachis oil treated) employed in the present studies exhibited characteristic differences of glucose tolerance and basal plasma insulin levels. Basal blood sugar levels were almost identical between the two sexes and although glucose tolerance appeared to be slightly greater in the male, as evaluated by the ipGT_A, this was not statistically significant. However, the shape of the glucose curve was distinctly different. Peak blood sugar levels determined at 15 minutes after glucose loading were significantly lower in the males than in the females ($p < 0.01$) and the generally more flattened curve of the male was associated with an almost linear decline of blood sugar from

15 to 60 minutes (fig.16).

The characteristic shape of the glucose tolerance curve was also evident in the intact male and female animals (fig.17) and in the hormone treated gonadectomized animals (figs 12 & 14) with one exception, the testosterone treated females. These animals showed a rise in blood sugar at 15 minutes characteristic of the female, but this was followed by an almost linear decline parallel to that of the male (fig.16). The possible implications of this observation are considered later. Basal plasma insulin levels in the control males were significantly lower than those in the control females (30.5 ± 1.9 versus 54.1 ± 4.5 ; $p < 0.001$).

It is inferred that in the absence of normal gonadal influences, the disposal of a glucose load is accomplished more efficiently in males than in females and this may be associated with greater insulin sensitivity in the former individuals.

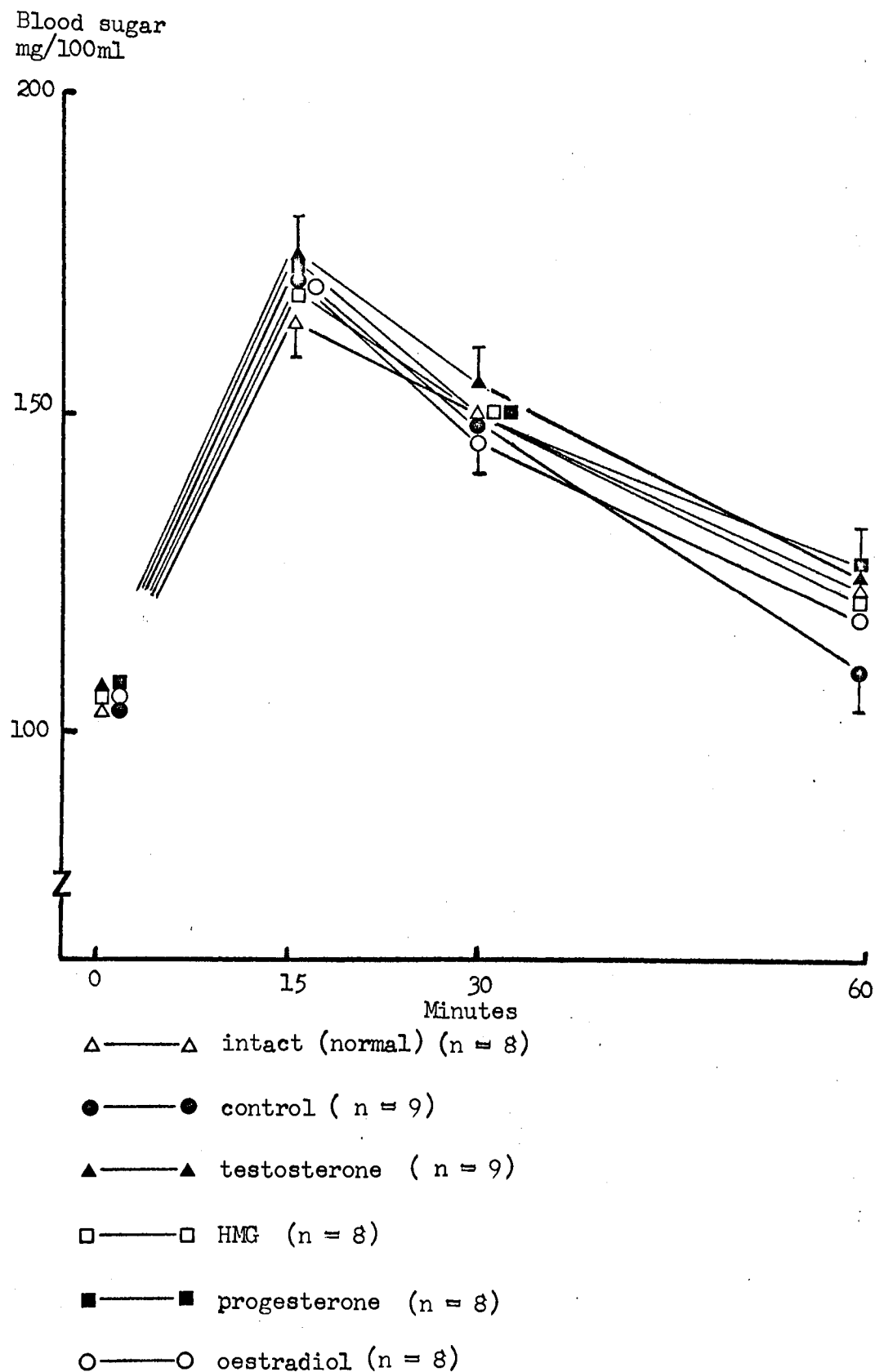
In the normal male and female rats the overall ability to deal with a glucose challenge is very similar, since the $ipGT_A$ of the male is within the range of $ipGT_A$ values exhibited by the females at different stages of the oestrous cycle (fig.17). A comparison of the basal plasma insulin levels in the normal males and females at different stages of the oestrous cycle may not be made since different batches of anti-porcine insulin antibody were used in the two sets of assays, and it is not known whether they possess the same avidity for rat insulins. Thus it appears that normal gonadal function reduces the differences of glucose tolerance that may be discerned in the gonadectomized state. This may reflect a beneficial influence of gonadal function on

carbohydrate metabolism in the female, since glucose tolerance tends to become improved, while there may be a marginal decline of glucose tolerance in the male. These influences are consistent with the effects of oestradiol and progesterone observed in ovariectomized females and with the effects of testosterone observed in castrated males.

It is tempting to speculate that the sex differences in the pattern of glucose tolerance may reflect the influence of different gonadal steroid environments in males and females, and that these patterns are not lost after steroid deprivation. The development of male glucose tolerance characteristics in testosterone treated ovariectomized females further suggests that the shape of the curve in the male might reflect the influence of this hormone.

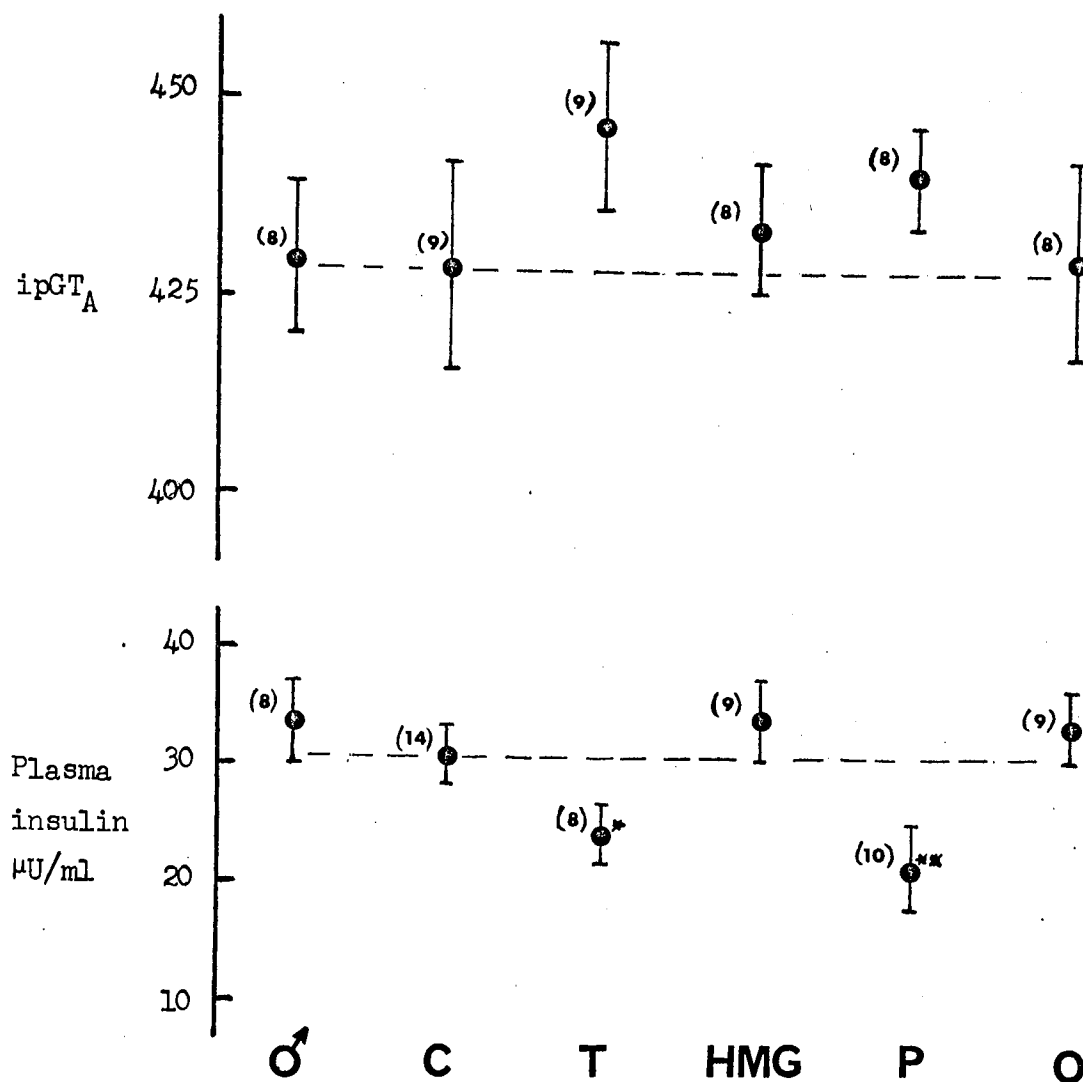
The different effects of sex hormones on glucose homeostasis in male and female rats indicate that there may be sex differences of the tissues involved in mediating these effects and of the nature of their responses to the hormones. This possibility is discussed further in chapter ten.

Figure 14 Intraperitoneal glucose tolerance in intact male rats and in orchidectomized male rats treated with testosterone, HMG, progesterone and oestradiol (mean \pm sem)



All steroid treatments at dosage 2.5 μ g/100g body weight
 HMG - 2.5iu FSH, 2.5iu LH/100g body weight

Figure 15 Intraperitoneal glucose tolerance area and basal plasma insulin level in intact male rats and in orchidectomized male rats treated with testosterone, HMG, progesterone and oestradiol (mean \pm sem)



♂ - intact (normal)

C - control

T - testosterone

HMG - gonadotrophin

P - progesterone

O - oestradiol

* $p < 0.05$; ** $p < 0.01$; all values compared with control

Number of observations in parentheses

All steroid treatments at dosage 2.5 μ g/100 g body weight

HMG - 2.5iu FSH, 2.5iu LH/100g body weight

Figure 16a Intraperitoneal glucose tolerance in gonadectomized male and female rats, and in testosterone treated gonadectomized females (mean \pm sem)

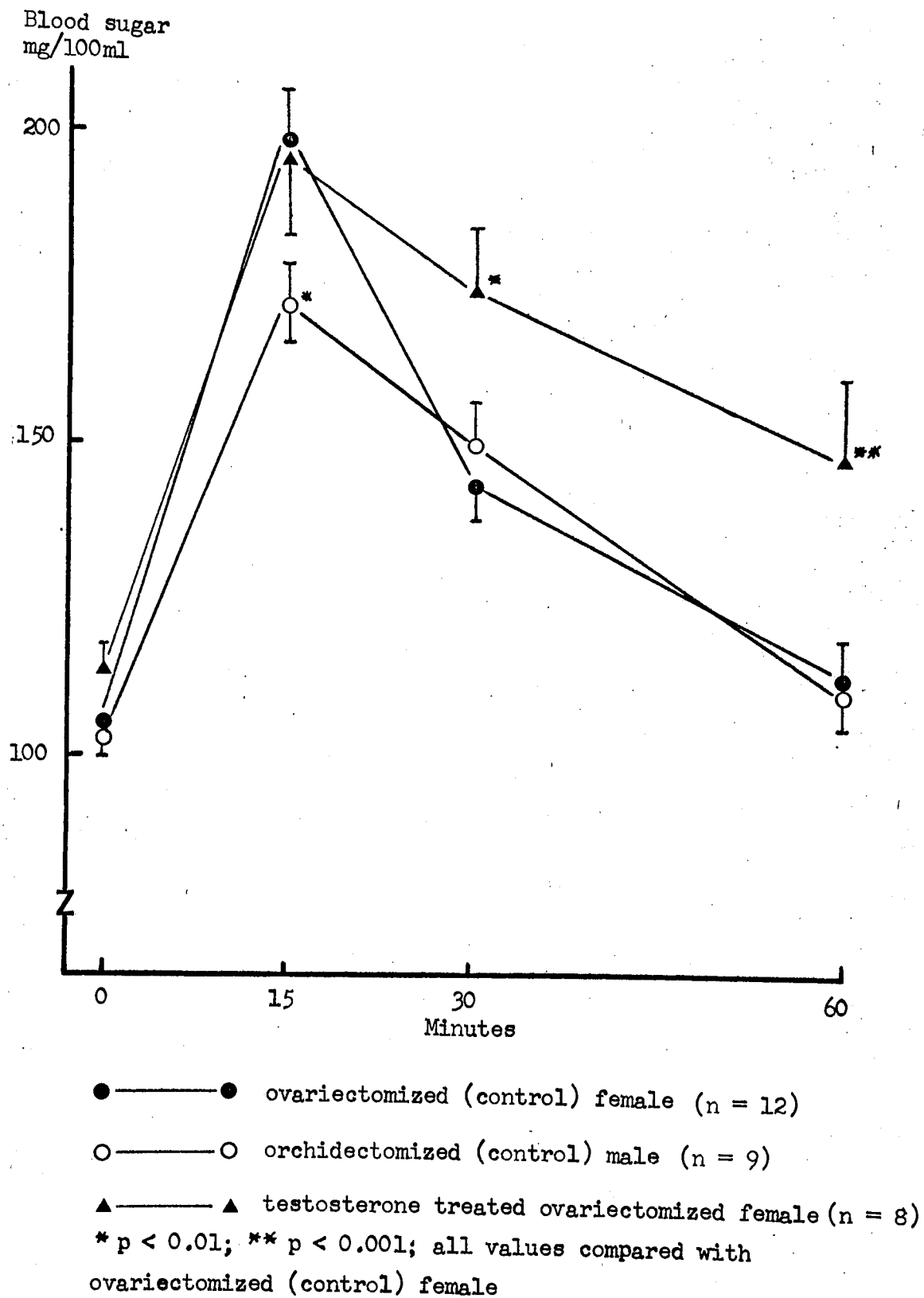
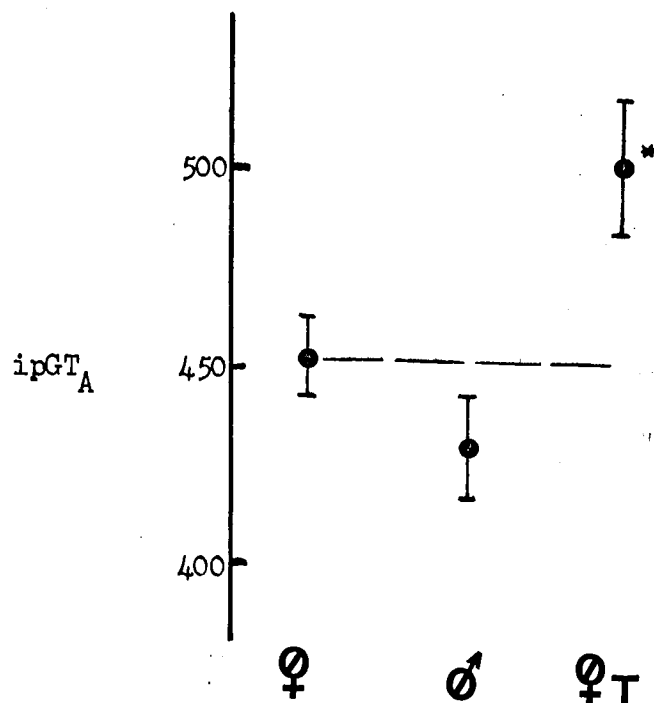


Figure 16b

Intraperitoneal glucose tolerance area in gonadectomized male and female rats, and in testosterone treated gonadectomized females (mean \pm sem)



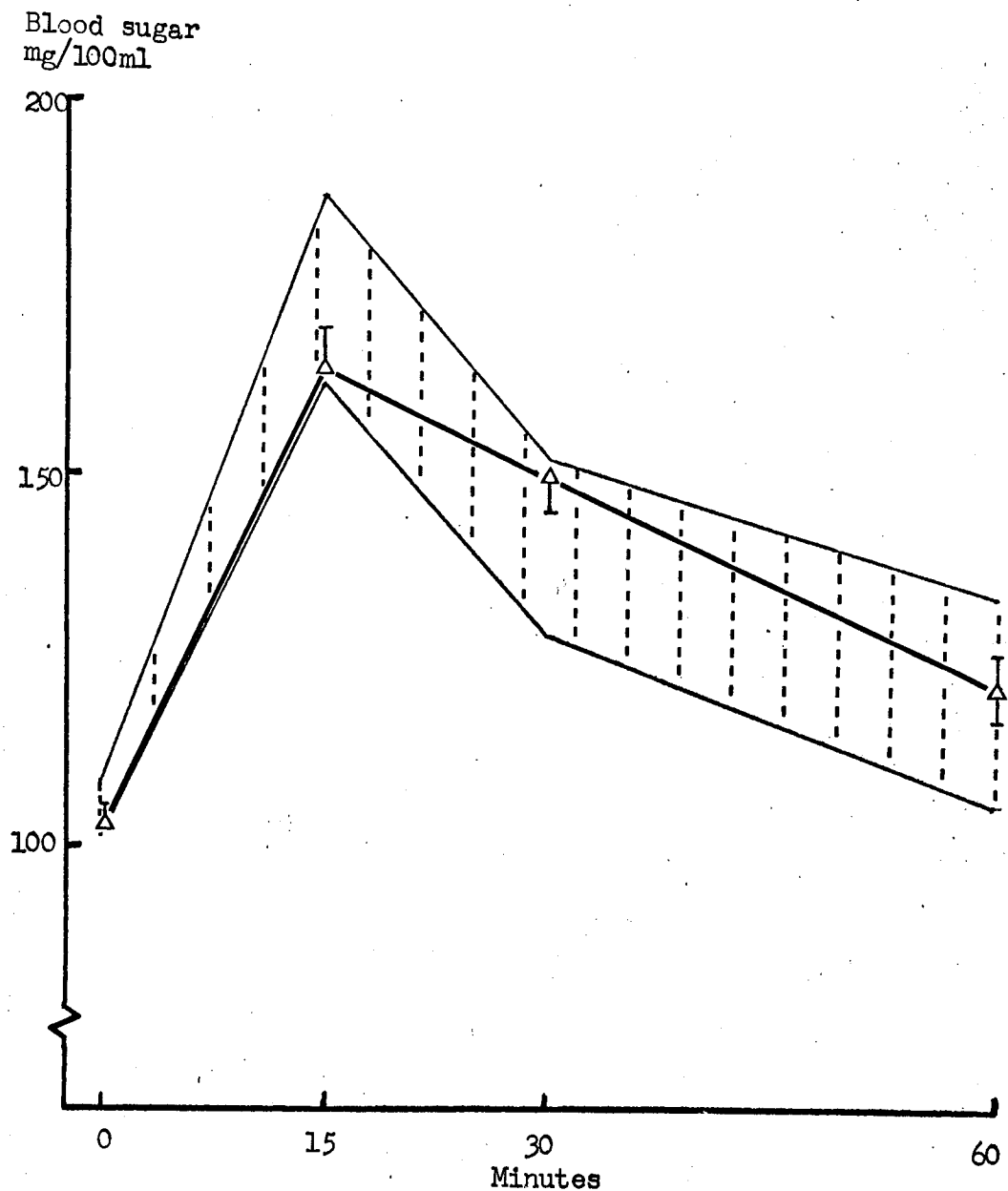
♀ ovariectomized (control) female (n = 12)

♂ orchidectomized (control) male (n = 9)

♀T testosterone treated ovariectomized female (n = 8)

* p < 0.05, compared with ovariectomized (control) female

Figure 17a Intraperitoneal glucose tolerance in intact male and female rats



Δ — Δ mean \pm sem value for intact (normal) males
(n = 8)

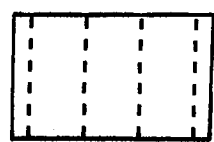
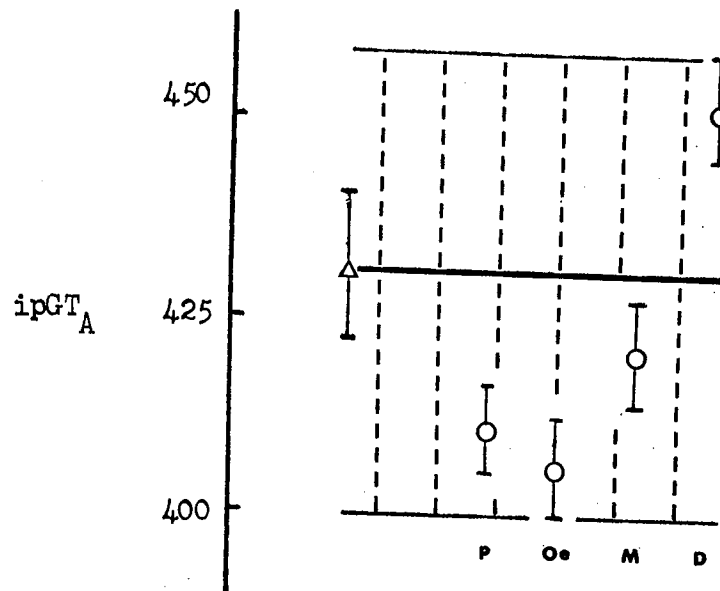
 range of mean \pm sem values for intact
females at different stages of the oestrous
cycle

Figure 17b Intraperitoneal glucose tolerance area in intact male and female rats



△ — △

mean \pm sem value for intact (normal) male (n = 8)

□

range of mean \pm sem values for intact females at different stages of the oestrous cycle

○

mean \pm sem values for intact females at pro-oestrus (P), oestrus (Oe), metoestrus (M) and dioestrus (D)

CHAPTER 8

EARLY EFFECTS OF OESTRADIOL AND
PROGESTERONE ON INSULIN SECRETION IN
FEMALE RATS

EARLY EFFECTS OF OESTRADIOL AND PROGESTERONE
ON INSULIN SECRETION IN FEMALE RATS

Introduction

Foregoing experiments have demonstrated the effects of certain sex hormones on glucose tolerance and basal plasma insulin levels following two weeks of treatment in gonadectomized male and female rats. It appears from these experiments that sex hormones do not produce the same effect in each sex, and furthermore oestradiol, progesterone and gonadotrophins show much greater activity in the female than testosterone and gonadotrophins show in the male. This suggests that sex hormones bear a greater influence on glucose homeostasis in the female than in the male.

The present investigation is concerned with the mechanisms through which sex steroids may be regulating glucose homeostasis in the female rat, and in particular the effects of these hormones on insulin secretion. Costrini & Kalkhoff (1971) demonstrated that following three weeks of treatment with oestradiol or progesterone in ovariectomized female rats the fasting plasma insulin level and the plasma insulin response to an intravenous glucose challenge were significantly elevated. Data presented herein have shown that ovariectomized female rats receiving oestradiol or progesterone for two weeks show a significant rise in the basal plasma insulin level. In both studies oestradiol and progesterone significantly improved glucose tolerance. Towards an understanding of the mechanisms

involved, the experiments reported in this chapter were designed on the basis of current concepts of the mode of action of steroid sex hormones.

Mode of action of steroid sex hormones

The cellular metabolism of sex steroids has formed the subject of several recent expositions (James & Fotherby, 1970; McKerns, 1971; O'Malley, 1971; Smellie, 1971; Williams-Ashman & Reddi, 1971; Jensen & DeSombre, 1972; Means & O'Malley, 1972; Mueller, Vonderhaar, Kim & Le Mahieu, 1972; Wilson, 1972; Thomas, 1973).

It is generally held that steroid sex hormones regulate specific gene expression. The primary control of the hormone appears to reside at the level of transcription which in consequence dictates the translational events that lead to the formation of new enzyme protein. The metabolic modifications induced within the cell are therefore effected through enzyme induction.

Further mechanisms of steroid action have been indicated. Gelehrter (1973) has reviewed evidence that enzyme induction may be controlled through antagonism of the action of a post-transcriptional repressor independently of the genomic control. It has also been suggested that sex steroids may produce rapid cellular effects through a cyclic AMP-dependent mechanism, since enhanced adenylyl cyclase activity and raised cyclic AMP-protein kinase levels have been observed immediately following steroid treatment (Robison, Butcher & Sutherland, 1971; Major & Kilpatrick, 1972). "Whether cyclic AMP or any other low

molecular weight substances serve as second messengers for the actions of estrogens, gestagens, or androgens ... remains a moot point" (Williams-Ashman & Reddi, 1971).

The constraints of protein biosynthesis delay the expression of sex hormone induced effects for at least two hours and a maximal effect of the hormone may not become apparent for a considerably greater period. Thus, the influence of sex steroids on physiological systems, which may involve secondary and tertiary effector tissues may not become detectable for several hours.

Present knowledge in this sphere has been derived largely from studies of hormone interaction with the so-called "target tissues" - tissues which preferentially accumulate comparatively large amounts of hormone, namely the secondary sex organs and certain regions of the hypothalamus. It is unlikely that sex steroid target tissues contribute an important role in blood glucose homeostasis, but this does not preclude an effect of sex steroids on this process: there are already several well documented examples of sex steroids entering and modifying the metabolic expression of non-target tissues by direct and indirect mechanisms (James & Fotherby, 1970; Williams-Ashman & Reddi, 1971).

Experimental design

The experiments described below were conducted during the early time course of steroid hormone action. Changes of insulin secretion occurring during the initial 30 minutes and between 6 and 6.5 hours following hormone administration have

been observed so that it is possible to distinguish between immediate and more slowly generated effects. The former effects are likely to reflect direct actions on the effector tissues, and are unlikely to involve a protein synthetic sequence. The latter effects are likely to incorporate these actions together with a genomic level of action, involving de novo protein synthesis and the excitation of secondary effector tissues.

Both in vivo and in vitro techniques have been employed. In this way it is possible to detect whether hormone induced changes of circulating plasma insulin (observed in vivo) are associated with a direct effect of the hormone on pancreatic insulin secretion.

HMG was not examined in these experiments since it was considered that further investigations with gonadotrophins warrant the use of preparations of guaranteed purity.

Materials and methods

To ensure an accurate representation of the early time course during which the steroid hormones are exerting their effects it is imperative that the steroid receptor mechanisms should be in the natural physiological resting state in all of the animals. The activity of steroid hormones and the levels of steroid receptor aggregates appear to fluctuate during

different reproductive states (Jensen & DeSombre, 1972; Thomas, 1973) and to eliminate the contra-effects of these phenomena previous investigators have usually employed gonadectomized animals. However, female rats that have been deprived of normal sex steroid titers for several days or longer (for example, following ovariectomy) show an abnormally delayed response to parenterally administered hormones, possibly due to the degeneration of tissue receptors (McGuire & Lisk, 1968; Ciaccio & Lisk, 1972). For the purpose of examining early actions of sex steroids in ovariectomized rats it has become customary to prevent this delay by administering a small (priming) dose of the appropriate hormone or combination of hormones up to 72 hours in advance of the actual experiment. Recently acquired evidence, however, indicates that even very small priming doses of steroid hormones produce high saturation of tissue receptors and this may reduce the effects of hormones that are administered during the experiment per se (James & Fotherby, 1970; Ciaccio & Lisk, 1972). In view of this evidence, hormone priming in advance of short term studies on steroid hormone action may prove to be a dubious practice.

The present study was conducted on intact dioestrus female rats. The dioestrus condition corresponds with normal basal endogenous sex steroid activity and provides a natural physiological environment in which the tissues may respond to hormonal stimulation. Furthermore, the endogenous levels of sex hormones that occur during earlier phases of the oestrous cycle serve as a natural physiological priming regime. For these reasons the intact dioestrus female may be considered a more

acceptable model for studying the early actions of sex steroids than the hormone primed ovariectomized animal.

In vivo studies

Animals of 10 weeks of age and weighing about 200 g were fasted during the night of metoestrus to dioestrus and modified intravenous glucose tolerance tests (page 34) were performed on the morning of dioestrus.

The first series of experiments were concerned with the immediate effects of sex steroids, and the hormones were administered by an intravenous injection into the jugular vein simultaneously with the glucose challenge. The hormones were dissolved in absolute ethanol (0.002 ml/100g body weight) and control animals received vehicle alone.

In the second series of experiments the hormones were administered by subcutaneous injection at exactly 6 hours prior to the glucose challenge. The vehicle used was arachis oil since this vehicle and the subcutaneous route of administration serve to reduce the rate of entry of hormone into the circulation. Control animals were treated with the vehicle alone.

Identical doses of hormones were administered in each series of experiments: 17 β -oestradiol, 10 μ g/100 g body weight and progesterone 1 mg/100 g body weight. Accurate measurements of the small volumes of hormone were achieved with an 'Agla' micrometer syringe.

In vitro studies

Non-fasted animals of similar age and weight to those used

in the in vivo studies were killed by a blow on the head on the morning of dioestrus. The pancreas was removed and transferred to a petri dish containing cold ($+ 2^{\circ}\text{C}$), oxygenated, low glucose (0.5 g/l) bicarbonate buffer (Gey & Gey, 1936), pH 7.4. The organ was washed, trimmed free of fat, lymph nodes and large blood vessels, and cut into about 100 pieces (each weighing approximately 10 mg).

The first series of experiments examined the effect of oestradiol or progesterone on insulin secretion during 30 minutes exposure to the pancreas pieces. The pieces were preincubated in 30 ml of low glucose medium (0.5 g/l) containing bovine serum albumin (2.0 mg/ml) in a 100 ml conical flask for 30 minutes. The flask was slowly oxygenated and shaken gently throughout this period.

Test incubations were then performed. Groups of 5 to 7 pieces of tissue were washed briefly in medium of composition similar to that in which they were subsequently to be incubated and transferred to 1 dram glass vials containing 2 ml of oxygenated test incubation medium at 37°C . The vials were oxygenated once more, stoppered, and incubated for 30 minutes at 37°C with continual gentle shaking. A 100 μl sample of the final medium was withdrawn and stored at -20°C until analysis for insulin. The tissue was removed, dried and weighed.

The test medium consisted of bicarbonate buffer, bovine serum albumin (2 mg/ml), soybean trypsin inhibitor (20 $\mu\text{g/ml}$), either no glucose or 3.0 g/l glucose, and either 17β -oestradiol

(20 $\mu\text{g/ml}$), progesterone (40 $\mu\text{g/ml}$) or steroid vehicle only (absolute ethanol to a concentration of 0.1%).

The second series of experiments examined the effects of oestradiol or progesterone on insulin secretion during 6.5 hours of exposure to the pancreas pieces. About 30 pieces of pancreas were randomly transferred to three 25 ml conical flasks containing 10 ml of oxygenated preincubation medium at 37°C. The medium comprised low glucose buffer (0.5 g/l) containing bovine serum albumin (2.0 mg/ml) as used for preincubation of tissue in the former series of experiments. To this was added either 17 β -oestradiol (20 $\mu\text{g/ml}$), progesterone (40 $\mu\text{g/ml}$) or vehicle only (absolute ethanol to a concentration of 0.1%). Preincubation was extended over 6 hours at 37°C with slow oxygenation and gentle shaking throughout. At hourly intervals the medium was carefully siphoned off with a pasteur pipette and immediately replaced with fresh oxygenated medium at 37°C. This procedure was adopted to maintain the levels of sex hormones and to prevent the accumulation of undesirable levels of metabolites in the medium.

At the end of the preincubation period 30 minute test incubations on groups of 5 to 7 pieces of tissue were performed as described previously: a 100 μl aliquot of the final test incubation medium was withdrawn for insulin analysis and the dry weight of the pancreas tissue was determined.

A sample of tissue which had undergone a 6 hour preincubation in the low glucose medium (0.5 g/l) followed by 30 minutes incubation in medium containing 3.0 g/l glucose was examined

histologically. Sections were stained with H.E. or Gomori's A.F. counterstained with light green. As shown in Plate B the islets appeared to possess normal structural integrity and normal amounts of A.F. positive staining granular material. These observations, in conjunction with observations that the tissue responds normally to glucose stimulation (see below) indicate that the 6.5 hour incubated preparation is suitable for metabolic studies.

The doses of hormones used in these studies represent several orders of magnitude above the estimated upper levels occurring in the plasma. In the female rat 5 ng/ml of oestrogen (in oestradiol equivalents) and 0.1 µg/ml of progesterone are recorded during pregnancy (Yoshinaga et al., 1969; Sutter-Dub et al., 1973).

Results

In vivo studies

The immediate effects of oestradiol and progesterone on the arterial blood sugar and plasma insulin levels following an intravenous glucose challenge are illustrated in figure 18. The hormones did not produce any significant alterations of either parameter. However, a tendency for both steroids to raise plasma insulin levels was apparent.

Tests performed 6 hours following sex hormone administration revealed significant alterations of both arterial blood sugar and plasma insulin levels (fig.19). The fasting plasma insulin level tended to be raised by each hormone, although this was only significant in the case of progesterone. Fasting blood

sugar levels were not significantly altered. Following a glucose challenge the blood sugar levels were significantly reduced in the oestradiol and progesterone treated groups, and the plasma insulin response was significantly enhanced.

In vitro studies

As shown in figure 20, oestradiol and progesterone did not alter insulin secretion during 30 minutes exposure to the pancreatic tissue in media containing either no glucose or 3.0 g/l glucose.

The steroids were also without effect on insulin secretion in the absence of glucose after 6.5 hours exposure to the tissue. However, in the presence of 3.0 g/l glucose, progesterone significantly increased insulin secretion while oestradiol was again without any apparent effect.

Discussion

These results indicate that oestradiol and progesterone possess the capacity to enhance plasma insulin levels and to reduce blood sugar levels in response to an intravenous glucose challenge within 6 hours. The elevated plasma insulin levels observed following progesterone administration may well be due, at least in part, to a direct insulinotrophic action, since this hormone significantly enhanced glucose induced insulin secretion in vitro during a similar period of time. Although oestradiol also elevated plasma insulin levels 6 hours following administration in vivo, this hormone appears to be acting entirely through indirect mechanisms since it does not exert an insulinotrophic effect in vitro during this time.

The changes of plasma insulin and blood sugar levels recorded 6 hours after the administration of oestradiol and progesterone were not apparent within 30 minutes. There was some indication that a slightly greater plasma insulin response to glucose stimulation may be brought about in vivo, but such an effect was not associated with a direct action of the hormones on pancreatic insulin secretion.

Although there are several reports in the literature that oestradiol and progesterone raise the circulating insulin pool and improve glucose tolerance, the early time course of this effect and the possible mechanisms involved have received little attention. With regard to the early time course, Beric et al. (1957) have demonstrated an improvement of oral glucose tolerance 2 hours following intramuscular injection of 5 mg of oestradiol dipropionate to normal women, but Talaat et al. (1965a) were unable to identify any alteration of intravenous glucose tolerance 12 hours after 25 mg intramuscular injection of the same preparation. Women receiving a 250 mg intramuscular injection of 17-hydroxyprogesterone caproate produced an elevated plasma insulin response to oral glucose during tests performed 12 hours later (Schreibman, 1968). There have been no previous indications of the early time course during which oestradiol and progesterone effect glucose tolerance and plasma insulin in experimental animals.

Costrini & Kalkhoff (1971) investigated a direct effect of oestradiol and progesterone on pancreatic insulin secretion in the female rat. These workers incubated the hormones with isolated islets in vitro for 2 hours and measured glucose

induced insulin secretion during the following hour. Insulin secretion was apparently unaffected by the hormones. It is difficult to equate their results with the present results since the two studies have a number of methodological differences, and Costrini & Kalkhoff (1971) did not state the reproductive phase of the animals used. However, by combining the two sets of data one might speculate that the direct insulintrophic effect of progesterone becomes manifest between 3 and 6 hours.

Recently Hager, Georg, Leitner & Beck (1972) have evaluated the insulin content and secretory activity of islets isolated from intact female rats receiving oestradiol or progesterone for 2 weeks. Earlier reports that these hormones significantly increase the insulin content of the islets were confirmed and it was noted that glucose induced insulin secretion was enhanced by the progesterone treatment but reduced by oestradiol treatment. To explain these apparently incongruous actions of oestradiol (increasing insulin synthesis and reducing insulin secretion) the authors make reference to the short, 10 minute, incubation period used to assess insulin secretion. Since this period corresponds mainly to the first phase of insulin secretion (Curry et al., 1968) the authors suggest that oestradiol suppresses the initial phase of insulin secretion although it may enhance secretion during the second phase. The in vivo experiments reported herein and by Costrini & Kalkhoff (1971) have shown that the plasma insulin level 10 minutes following an intravenous glucose injection is considerably elevated in female rats treated with oestradiol for 6 hours and 3 weeks respectively. These observations suggest that oestradiol does not suppress the initial phase of insulin secretion

in vivo.

The early time course during which oestradiol and progesterone produce detectable changes of blood sugar and plasma insulin levels offers some indication of the likely mechanisms involved. A tendency of the hormones to produce an immediate though only slight rise in the plasma insulin response to glucose opens up the possibility of an effector sequence that does not include de novo protein synthesis. However, the mild and transient nature of this response, even at the high doses of hormone employed, suggests that its physiological importance may be negligible. Since more distinctive effects of sex hormones appear later it is likely that other mechanisms are of greater significance and a genomic level of action and the excitation of secondary effector tissues seems probable.

Figure 18a Immediate effects of oestradiol and progesterone on intravenous glucose tolerance (mean \pm sem).

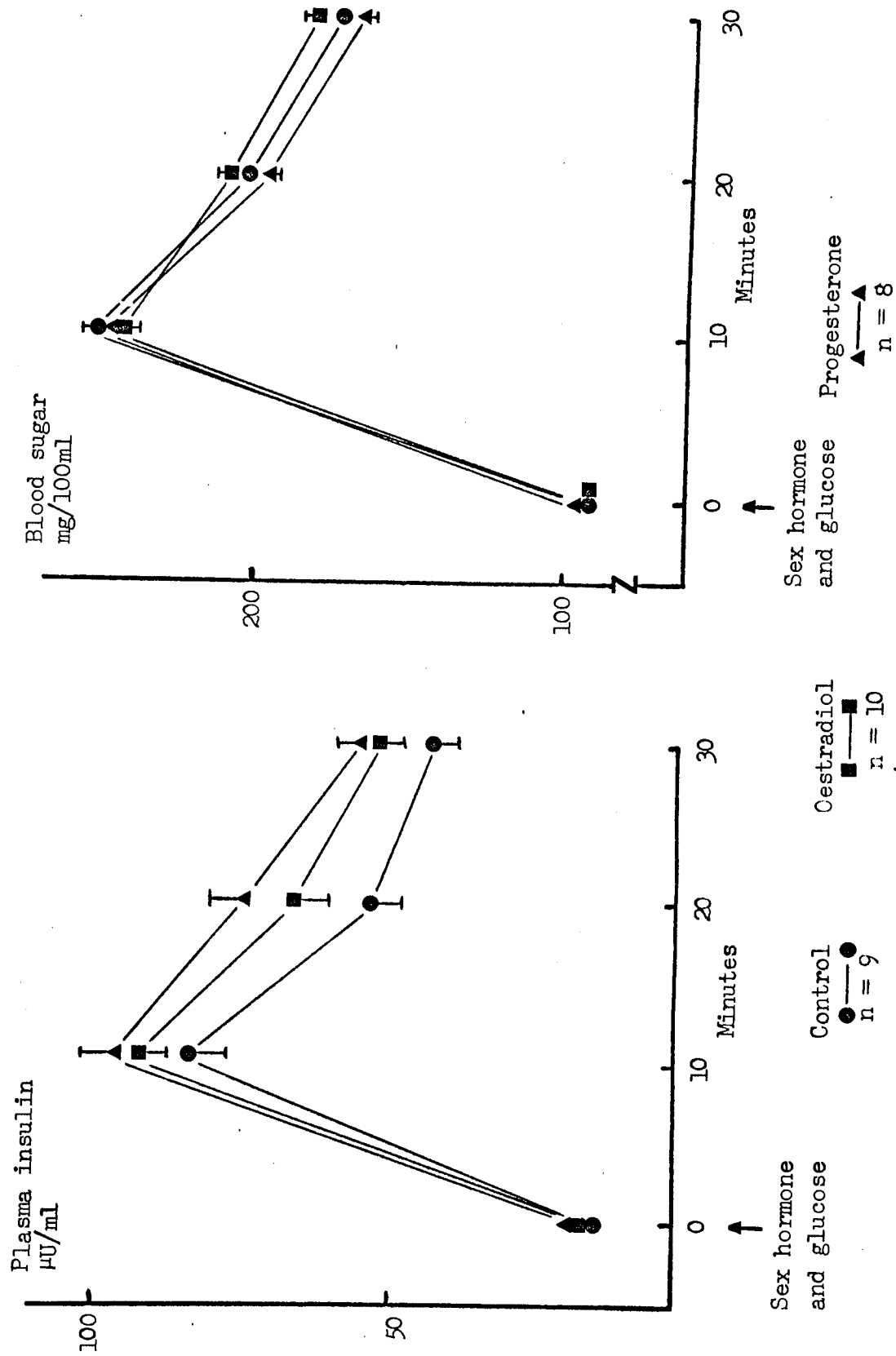
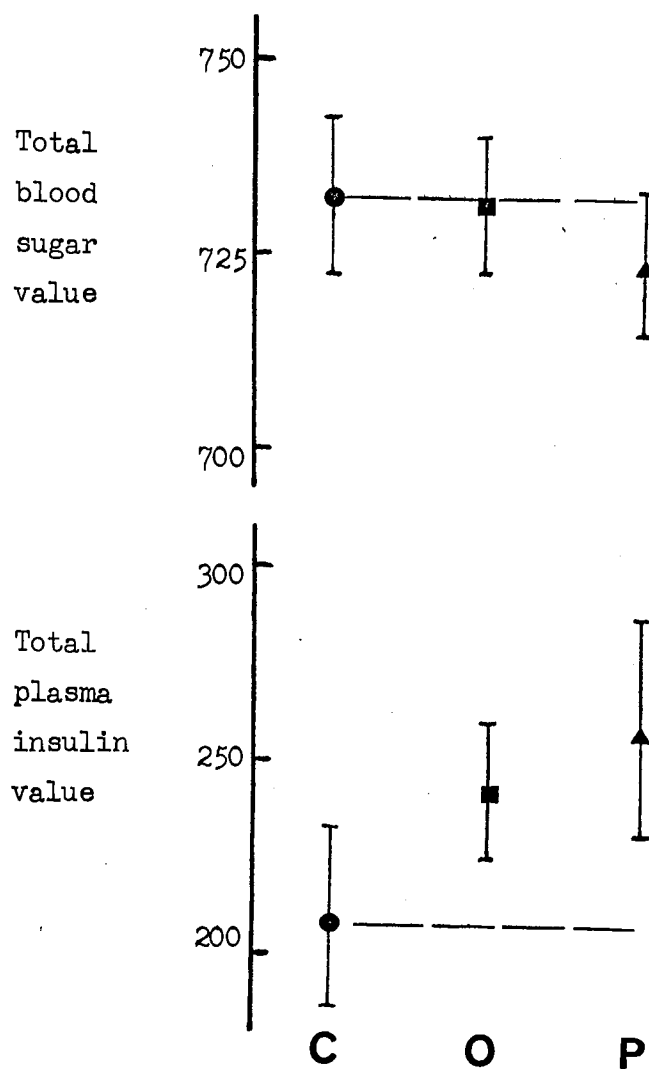


Figure 18b Immediate effects of oestradiol and progesterone on the total blood sugar and plasma insulin values during intravenous glucose tolerance tests (mean \pm sem)



C - control (n = 9)

O - oestradiol (n = 10) (10 μ g/100g body weight)

P - progesterone (n = 8) (1 mg/100g body weight)

Figure 19a Six hour effects of oestradiol and progesterone on intravenous glucose tolerance (mean \pm sem)

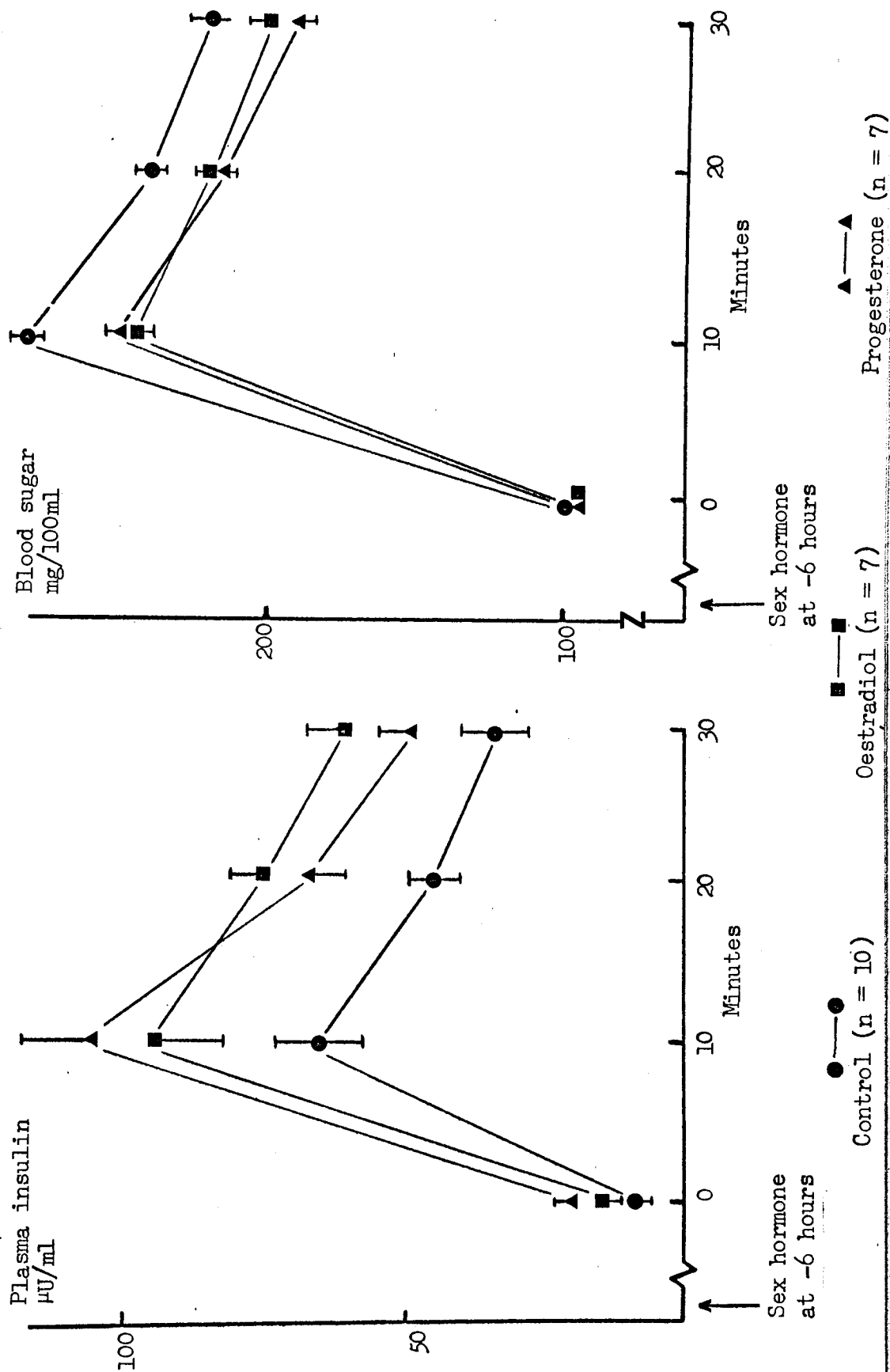
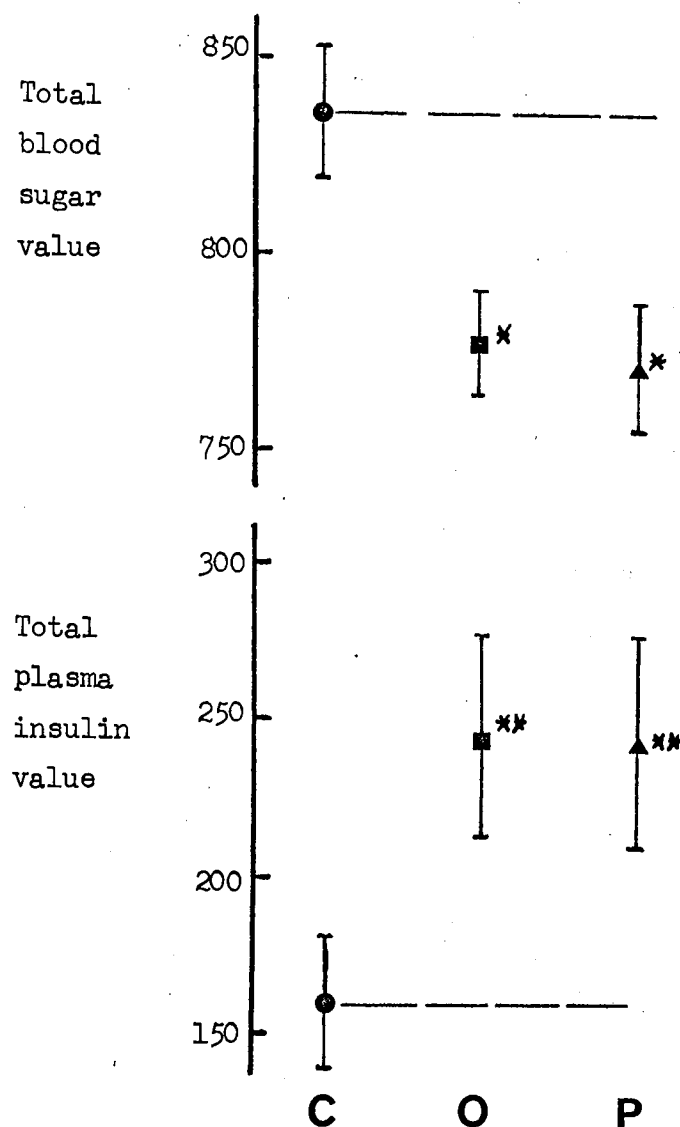


Figure 19b Six hour effects of oestradiol and progesterone on the total blood sugar and plasma insulin values during intravenous glucose tolerance tests (mean \pm sem)



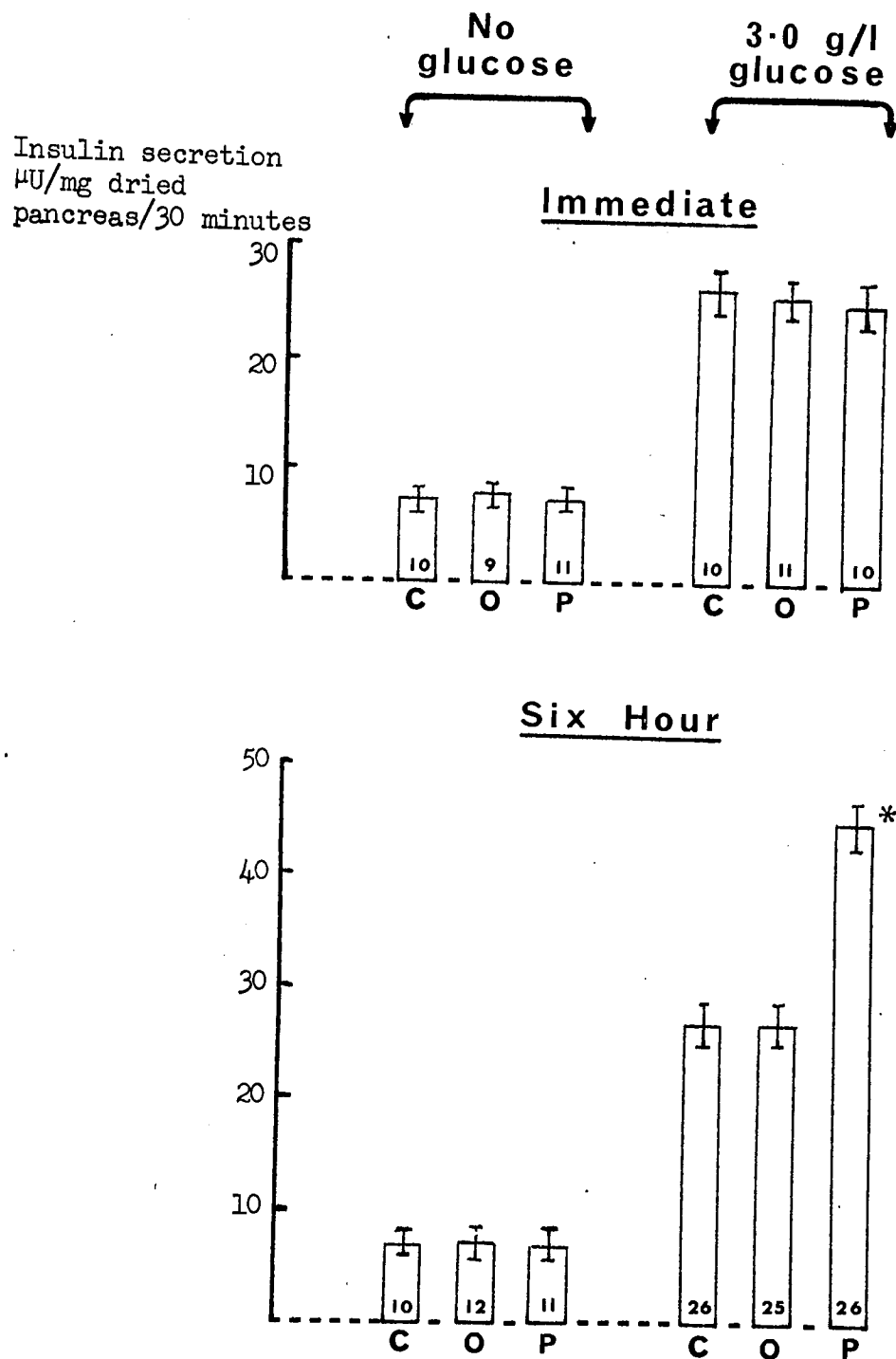
C - control (n = 10)

O - oestradiol (n = 7) (10 μ g/100g body weight)

P - progesterone (n = 7) (1 mg/100g body weight)

* p < 0.02; ** p < 0.05; all values compared with control

Figure 20 Immediate and six hour effects of oestradiol and progesterone on insulin secretion in vitro (mean \pm sem)



C - control

O - oestradiol (20 µg/ml)

P - progesterone (40 µg/ml)

* $p < 0.02$, in comparison with control

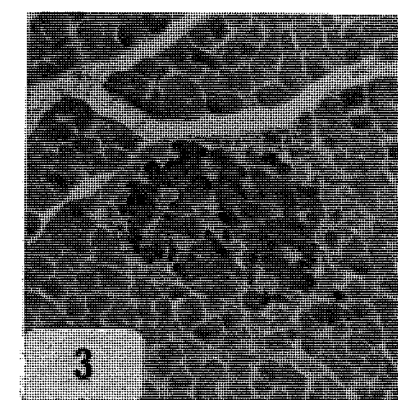
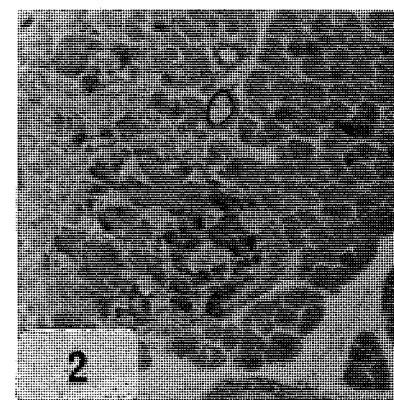
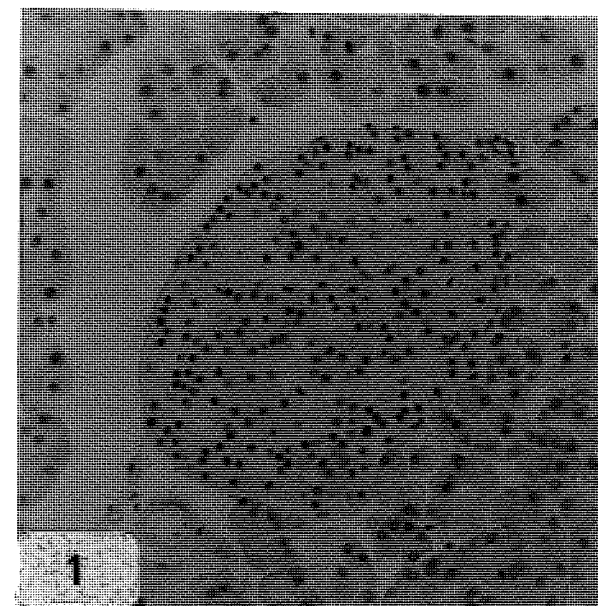
Number of observations at foot of bar

Description of Plate B

Islets of Langerhans in pancreas pieces preincubated for
6 hours in low glucose medium followed by 30 minute
incubation in high glucose medium

1 Islet stained with H.E. (x 50).

2,3 Islets stained with A.F. (x 50).



CHAPTER 9

EFFECTS OF MELATONIN ON INSULIN
SECRETION IN MALE RATS

EFFECTS OF MELATONIN ON INSULIN SECRETION
IN MALE RATS

Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a biogenic amine uniquely synthesised by the pineal gland. Recent studies have elucidated an important role of the pineal gland in the control of reproduction (see Wurtman, Axelrod & Kelly, 1968; Wurtman, 1969). The pineal gland functions as a "neuroendocrine transducer organ", translating neural inputs from the eyes and higher autonomic centres into an endocrine output in the form of melatonin and possibly other biogenic amines, which modify gonadal activity. Melatonin is synthesised under the influence of darkness and causes a suppression of gonadal function through an action at the hypothalamic level and directly on the gonadal tissue. It appears that melatonin is intimately involved in the regulation of the reproductive cycle of the female.

Two recent reports have suggested that the pineal gland may influence carbohydrate metabolism. Csaba & Barath (1971) observed a greater rise in blood sugar following alloxan treatment in pinealectomized rats than in normal rats and concluded that the pineal reduces the activity of the beta cells. Milcu, Nanu & Milcu (1971) observed a greater plasma insulin response to intravenous glucose in pinealectomized rats than in normal rats and concluded the reverse. Thus an effect of the pineal on carbohydrate metabolism remains uncertain.

The present investigation concerns the effect of melatonin on the blood sugar and plasma insulin response to an intravenous glucose challenge and on insulin secretion in vitro.

Materials and Methods

Insulin secretion in vitro

Insulin secretion was evaluated in vitro using a preparation of pancreas pieces. Non-fasted male Wistar rats weighing about 200 g were killed by a blow on the head and the splenic portion of the pancreas was removed. Tissue from 3 rats was pooled for each experiment. The tissue was washed in oxygenated low glucose (0.6 g/l) buffer at 2°C, cleaned of lymph nodes, fat and large blood vessels and cut into approximately 150 pieces. The pieces were preincubated in 15 ml of low glucose (0.6 g/l) buffer for 20 minutes at 37°C with continuous oxygenation and shaking. Groups of 5 or 6 pieces were test incubated for 60 minutes in 2 ml of buffer containing 0 and 3.0 g/l glucose, with and without melatonin (100 µg/ml). Gentle oxygenation and shaking were maintained throughout this period. At the end of the experiment a 100 µl sample of the incubation medium was withdrawn for insulin analysis and the tissue was dried and weighed.

Data obtained using pancreas pieces was supplemented with an observation of the effect of melatonin on insulin secretion from an isolated perfused pancreas preparation. Non-fasted male Wistar rats weighing about 300 g were prepared as described later. Melatonin was infused at concentrations of 0.1 and 0.5 µg/ml in media containing 1.0 g/l glucose.

Intravenous glucose tolerance tests

Male Wistar rats weighing about 300 g were prepared for modified intravenous glucose tolerance tests (page 34). After a 10 minute stabilization period experimental animals received a constant infusion of melatonin (10 μ g/0.02 ml saline/minute), and control animals received a constant infusion of saline only (0.02 ml/minute). The infusion was delivered via the jugular cannula using an injection apparatus (C.F. Palmer Ltd., London). A rapid intravenous injection of glucose was given at time 10 minutes and blood samples (0.5 ml) for blood sugar and plasma insulin analysis were withdrawn from the carotid cannula at 0, 10, 20, 30 and 40 minutes.

Dilution of melatonin

For incubation studies with pancreas pieces and for the glucose tolerance tests melatonin was dissolved in a minimum of absolute ethanol and diluted in the appropriate solution: control solutions contained absolute ethanol only. During pancreas perfusion experiments the required amount of melatonin was dissolved in about 50 μ l of absolute ethanol and diluted in 1 litre of buffer. The buffer was temporarily stored to allow the absolute ethanol to evaporate.

Results

Insulin secretion *in vitro*

As shown in figure 21 melatonin (100 μ g/ml) significantly depressed insulin secretion from pieces of rat pancreas both in the presence and in the absence of glucose.

Melatonin (0.1 and 0.5 μ g/ml) in media containing 1.0 g/l

glucose also produced a small reduction of insulin secretion in the isolated perfused pancreas (fig.22).

Intravenous glucose tolerance tests

The mean blood sugar and plasma insulin values of groups of rats undergoing intravenous glucose tolerance tests during saline infusion or during melatonin infusion (10 μ g/min) are illustrated in figure 23. Plasma insulin levels were slightly (though not significantly) lowered at 10 and 20 minutes after glucose loading in the group receiving melatonin; but there were no apparent differences in blood sugar.

Discussion

The pineal gland is not only an important source of melatonin; it is also rich in other biogenic amines, such as serotonin, dopamine, noradrenaline and histamine. Supra-physiological doses of these agents have been shown to inhibit glucose induced insulin secretion in several species (Feldman & Lebovitz, 1970; Feldman, Boyd & Lebovitz, 1971; Feldman & Lebovitz, 1971; Quickel, Feldman & Lebovitz, 1971a), and fluorescent staining techniques have identified the presence of biogenic amines in the pancreatic islets of many species (Cegrell, 1968). A particularly abundant monamine pool was observed in the islets of the golden hamster and Feldman, Lebovitz and co-workers (Feldman & Lebovitz, 1971, 1972; Feldman, et al., 1971) have used this model for a systematic analysis of the effects of these agents on insulin secretion in vitro. These workers have postulated that the aliphatic amine group is essential for the inhibitory action, while the hydroxyl groups on the aromatic ring greatly enhance the inhibitory effect, but are not an

essential requirement (fig.24). These groups are well substituted in melatonin; and in line with their hypothesis Feldman & Lebovitz (1972) were unable to identify an inhibitory effect of melatonin at a concentration of 2×10^{-4} or $2 \times 10^{-3} M$ on glucose induced insulin secretion from pieces of golden hamster pancreas.

The data reported in this chapter demonstrate that melatonin (100 $\mu g/ml$, which is approximately $4.3 \times 10^{-4} M$) inhibits insulin secretion from pieces of rat pancreas. Further studies conducted in this department by Dr. Atkins have shown that the same concentration of melatonin significantly inhibits insulin secretion from isolated islets of obese hyperglycaemic mice and their lean littermates (Bailey, Atkins & Matty, 1973). The difference between these results and those of Feldman & Lebovitz (1972) may reflect differences of methodology or a species variation of either the sensitivity to melatonin or of the requirements for monamine inhibition of insulin secretion.

Melatonin appears to modify the activities of several other endocrine organs. It suppresses the release of hypophysial regulating hormones from the hypothalamus, which results in an impairment of the secretion of anterior pituitary hormones that are predominantly controlled by "releasing" hormones, but promotes the secretion of hormones predominantly controlled by "inhibitory" hormones (Gramova, Kraus & Krecek, 1967; Fraschini, Collu & Martini, 1971; Kamberi, Mical & Porter, 1971; Singh & Turner, 1972). A direct inhibitory action of melatonin on the testes (Debeljuk, Vilchez, Schnitman, Paulucci & Feder, 1971; Ellis, 1972) and on the ovaries (Wurtman, Axelrod & Chu, 1963)

has also been observed. The amount of melatonin present in the pineal gland is very low (Wurtman et al., 1968) and a sensitive and specific assay for melatonin in mammalian blood is not currently available. However, it has been shown that circulating melatonin is rapidly taken up by almost all tissues (Kopin, Parc, Axelrod & Weissbach, 1961).

A constant infusion of melatonin (10 μ g/minute) did not produce any changes in blood sugar and produced only a marginal depression of plasma insulin levels following an intravenous glucose challenge in rats. These results indicate that either insulin secretion per se was not significantly depressed by the dose of melatonin used in vivo, which may be explained by its extremely short biological half-life, or melatonin produces alterations in other systems which offset a depression of insulin secretion.

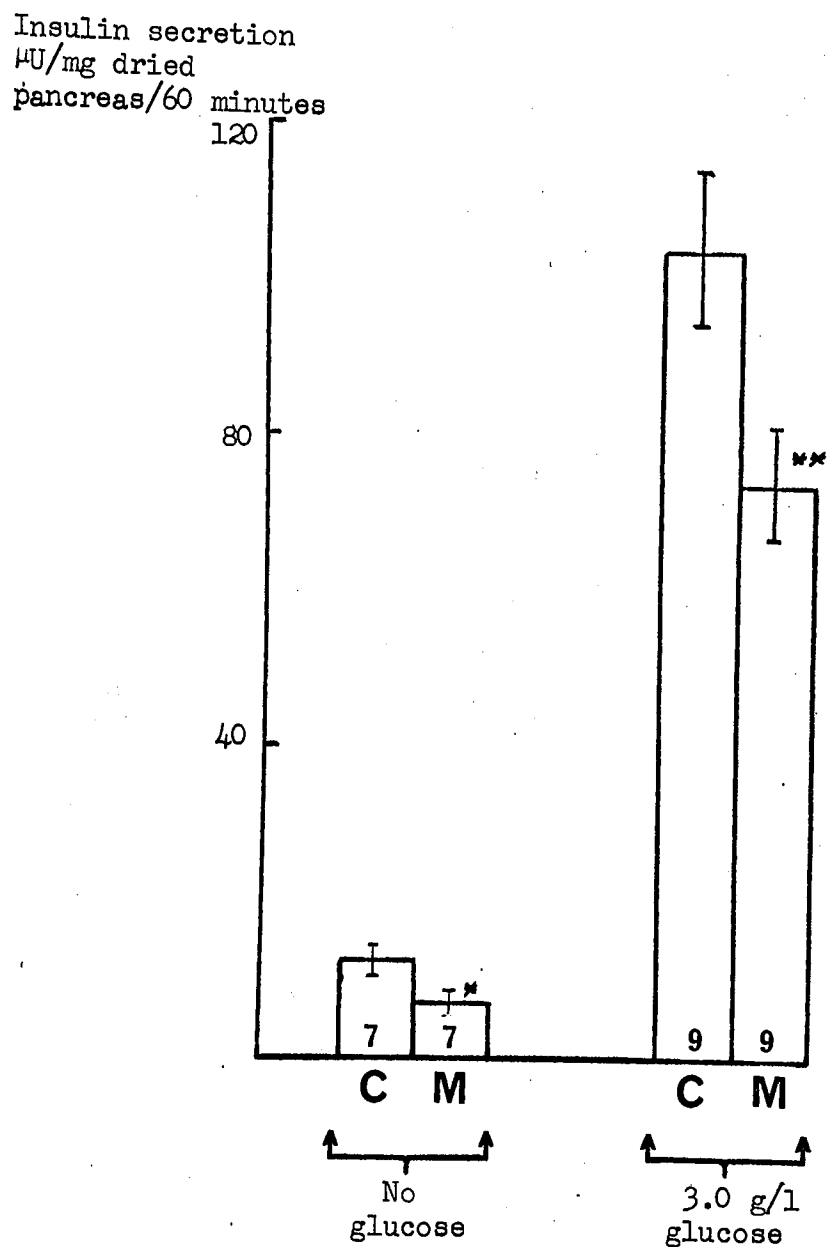
It has been shown that beta cells take up biogenic amines and their precursors from the blood (Cegrell, 1968) and there is extensive evidence for the involvement of monoaminergic mechanisms in both the normal physiological regulation of insulin secretion (Porte, 1969; Lundquist, 1971; Feldman, Quickel & Lebovitz, 1972; Feldman & Lebovitz, 1973) and the pathogenesis of maturity-onset diabetes (Quickel, Feldman & Lebovitz, 1973).

The mechanisms of melatonin inhibition of insulin secretion in the rat is unknown but it is possible that mechanism suggested for other monoamines might be applicable; such as stimulation of the alpha adrenergic receptors (Porte, 1969), depression of

lysosomal acid amyloglucosidase activation (Lundquist, 1971) and/or binding with the beta granules to impede normal emiocytosis (Hellman, Lernmark, Sehlin & Taljedal, 1972).

One may only speculate on the physiological significance of these effects of melatonin since the doses administered were presumably very high. It is possible that the small amounts of endogenously secreted melatonin are influencing insulin secretion through a direct effect on the pancreatic islets and also indirectly through an effect on other endocrine glands.

Figure 21 Effect of melatonin on insulin secretion from pieces of pancreas (mean \pm sem)



C - control

M - melatonin (100 μ g/ml)

*p < 0.02; ** p < 0.05; all values compared with control

Number of observations at foot of bar

Figure 22 Effect of melatonin on insulin release from an isolated perfused pancreas preparation in the presence of glucose, 100mg/100ml

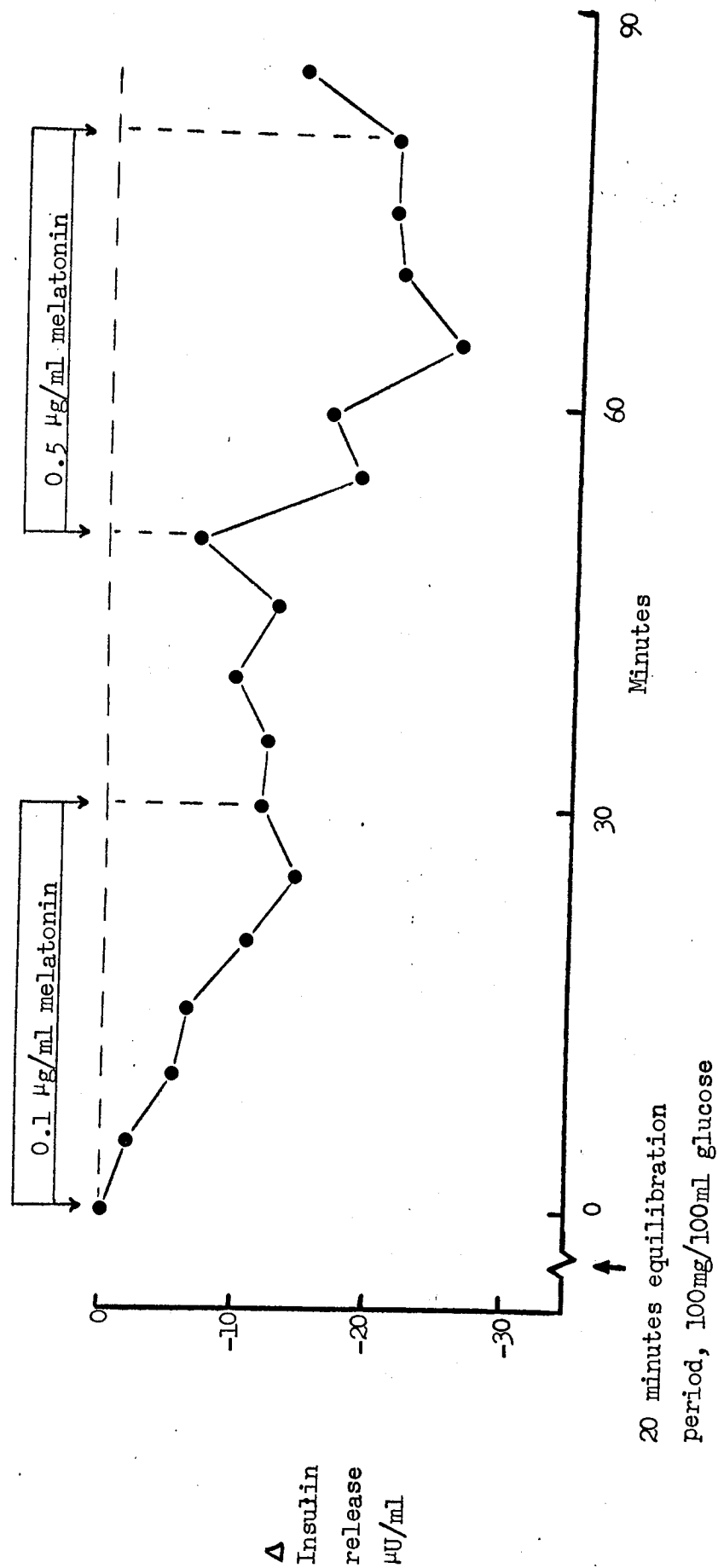


Figure 23 Effect of a constant infusion of melatonin on the arterial blood sugar and plasma insulin levels following an intravenous glucose challenge (mean \pm sem)

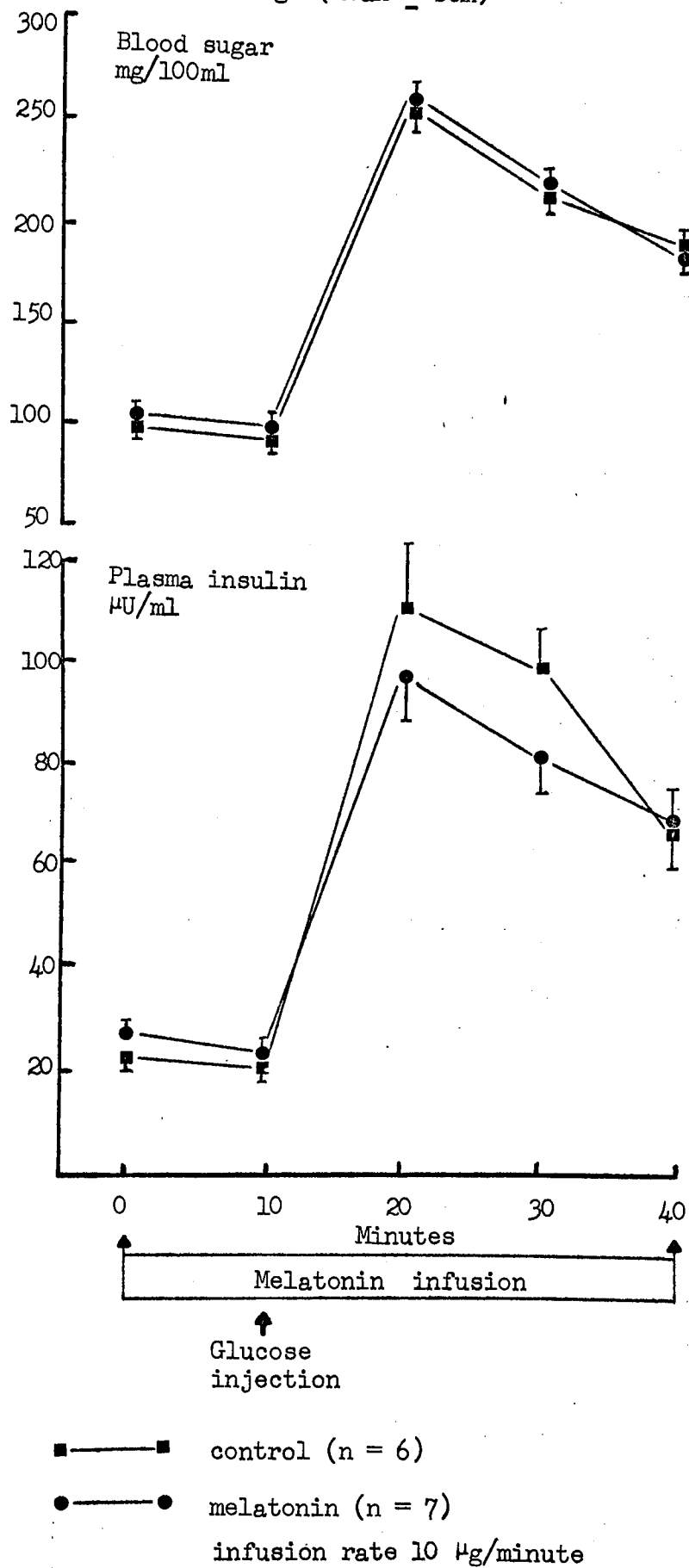
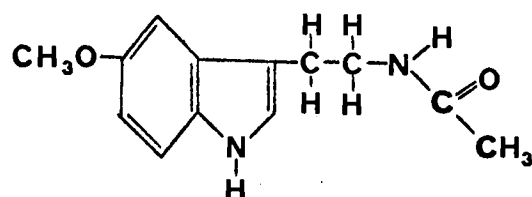
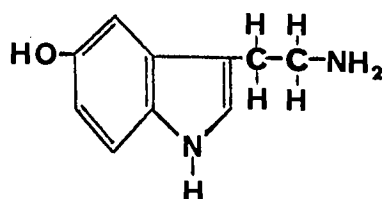


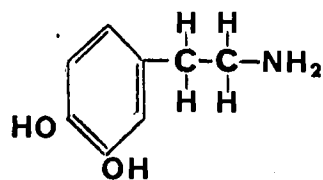
Figure 24 Structural features of some biogenic amines



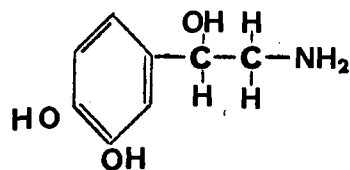
Melatonin



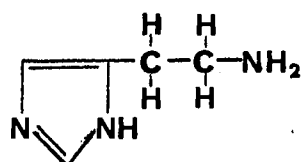
Serotonin



Dopamine



Noradrenaline



Histamine

CHAPTER 10

GENERAL DISCUSSION

GENERAL DISCUSSION

With respect to the general hypothesis that 'sex hormones participate in the physiological regulation of carbohydrate metabolism' it is not yet possible to construct a complete description of this process, but the evidence presented in this volume and in previously published reports may be collated to develop an appreciation of certain aspects of the whole.

Sex hormones appear to be intimately involved in the regulation of blood glucose homeostasis in the female rat. Oestradiol, progesterone and possibly gonadotrophins improve glucose tolerance and raise plasma insulin levels. The steroid hormones produce distinctive effects within six hours and progesterone exerts a direct effect on glucose induced pancreatic insulin secretion during this period.

Testosterone and gonadotrophins produce little alteration of the blood sugar level in the male rat, although testosterone shows a hypoinsulinaemic tendency. Sex hormones do not exert identical effects in males and females, and sex differences of glucose metabolism may be distinguished.

In this chapter the influence of sex hormones on circulating insulin levels and blood glucose homeostasis is evaluated in relation to the mechanisms which might be involved and their possible importance during different reproductive states in the female. No apology is made for the largely

speculative nature of this discussion since this approach illuminates areas for future investigation.

Aspects of the regulation of blood glucose homeostasis and the role of insulin have been extensively reviewed on several recent occasions. It is generally agreed that insulin secretion is controlled through the interaction of neural and hormonal mechanisms, and through the circulating levels of various substrates, cations and products of metabolism (Mayhew, Wright & Ashmore, 1969; Grodsky, 1970; Malaisse, 1972). The involvement of insulin and other hormones, neural factors, and the balance between different substrates and metabolites in the maintenance of blood glucose homeostasis are also generally appreciated (Hales, 1967; Dickens et al., 1968; Randle, 1970; Szabo & Szabo, 1972), but the intricate details of the co-ordination process await further elucidation.

Interaction between sex hormones and glucose homeostasis.

Several mechanisms through which sex hormones might influence the levels of plasma insulin and blood glucose are envisaged.

Plasma insulin levels might be influenced by:

(i) alteration of

- a) insulin secretion,
- b) insulin metabolism or degradation by the tissues,
- c) plasma protein binding of insulin,
- d) circulating insulinases,
- e) blood glucose levels,
- f) circulating levels of various other substrates and metabolites,

(ii) alteration of these phenomena via

- a) other hormones,
- b) neural mechanisms.

Blood glucose levels might be influenced by:

(i) alteration of

- a) factors concerned with the intake of glucose, e.g.
appetite, gastro-intestinal glucose absorption,
- b) gluconeogenesis,
- c) glycogenolysis,
- d) cellular uptake and metabolism of glucose,
- e) circulating levels of various other substrates
and metabolites,

(ii) alteration of these phenomena via

- a) other hormones,
- b) neural mechanisms.

A schematic representation of these possibilities is presented in figure 25.

Female rat

Evidence for the operation of these mechanisms in the female rat is very limited.

Insulin secretion

In vitro incubation studies suggest that progesterone promotes glucose induced insulin secretion by a direct action on the beta cells of the pancreatic islets, and the time course of this effect favours a genomic level of action. On the basis of this interpretation one might postulate that insulin biosynthesis

and insulin release are enhanced through induction of the enzymes involved in these processes (Steiner, Kemmler, Clark, Oyer & Rubenstein, 1972; Randle & Hales, 1972).

It is possible that progesterone modifies beta cell metabolism through the glucoreceptor mechanisms postulated by Randle and co-workers (1968) since this steroid did not influence insulin secretion in the absence of glucose. A further possibility is that progesterone acts via the acid amyloglucosidase system recently elaborated by Lundquist (1971, 1972a,b,c). This system depends upon the liberation of the enzyme acid amyloglucosidase from lysosomes within the beta cell. The enzyme promotes hydrolysis of glycogen to glucose and hence the stimulation of insulin secretion. Lundquist (1972c) noted that 15 to 20 minutes following an intraperitoneal injection of progesterone to female mice there was a marginal elevation of the plasma insulin level, but sulphonylurea induced insulin secretion was significantly enhanced by the progesterone pretreatment. He suggests that progesterone may act as a lysosomal labilizer to facilitate the liberation of acid amyloglycosidase by certain other insulinogenic factors. This process is likely to be rapidly effective. Since progesterone did not produce a detectable alteration of insulin secretion during 30 minute incubations in vitro it is assumed that the system did not contain such factors. Progesterone might conceivably stimulate insulin secretion through the adenyl cyclase system, but this system is associated with a quick

response (Malaisse et al., 1967b; Montague & Howell, 1972; Charles, Fanska, Schmid, Forsham & Grodsky, 1973) which was not apparent in the present study.

Further experiments are required to confirm a direct action of progesterone on the beta cells, and an effect mediated via other secretions of the pancreatic islets cannot be discounted: glucagon and gastrin are believed to provide a physiological control for insulin secretion, and biogenic amines released by certain other islet cells may also modulate insulin secretion.

Progesterone treatment was associated with an immediate non-significant elevation of the plasma insulin response to glucose. The in vivo environment may therefore contain factors which facilitate a rapid and direct insulintrophic effect.

Alterations of insulin secretion following progesterone administration in vivo are likely to involve both direct and indirect mechanisms. Synergism and antagonism between hormones are phenomena of increasing appearance to endocrinologists, for example: Sun, Lin & Haist (1972) have recently demonstrated that growth hormone and thyroxine act synergistically to enhance insulin biosynthesis. Progesterone may synergise or antagonise the actions of other hormones on insulin secretion.

There is little evidence to indicate that progesterone alters the circulating levels of other hormones which modulate insulin secretion. A single report by Bhatia, Moore & Kalkhoff (1972) suggests that progesterone suppresses the release of growth hormone in humans.

Oestradiol does not appear to exert a direct influence on pancreatic insulin secretion within six hours although it enhances the plasma insulin response to glucose within this period. An indirect mode of action of this hormone is thus implicated.

Oestrogens probably modify the activities of several endocrine moieties which are recognised regulators of insulin secretion: growth hormone secretion is inhibited in the rat (although the reverse influence has been observed in humans) (Catt, 1971), and changes in the circulating level of thyroxine and adrenal corticoids have been indicated without being conclusively established (Kitay, 1963; Ingbar & Woebar, 1969; Hinsull, 1972). Evidence such as this is not sufficient for any conclusions to be drawn.

Both oestrogens and progestins modify certain neural functions (Salhanick, Kipnis & Vande Wiele, 1969; Donovan, 1970) and influence electrolyte balance (Liddle, 1969): insulin secretion might be affected through these mechanisms.

It is likely that insulin secretion is modified through the effects of sex steroids on the circulating levels of various substrates and metabolites, but there is only meagre evidence to support this view: this is considered below.

Glucose-insulin inter-relationship

An effect of sex steroids on the biological half-life of insulin through plasma protein binding or enzymatic degradation has not been investigated although these hormones are known to

elevate the levels of corticoid binding globulin and thyroid binding globulin (Gala & Westpal, 1965; Williams, 1969; Catt, 1971).

Oestrogens appear to influence aspects of intermediary metabolism in several tissues: a synergistic action with insulin to promote the uptake of glucose by adipose tissue (Gilmour & McKerns, 1966); an enhancement of glucose uptake by the metrial muscle layers of the uterus (Warren, 1971); and an increased formation of liver glycogen through the facilitation of gluconeogenesis and the depression of glucose release (Matute & Kalkhoff, 1973) have been observed in the female rat. Several studies have been conducted on the effects of oestrogens on circulating lipid levels in women, but the results are not in general agreement (Furman, 1969; Kitao, 1971; Borenstein, Stahl, Usaf & Greenblatt, 1972).

In contrast to oestradiol, progesterone tends to antagonise the insulin dependent uptake of glucose by adipose tissue (Gilmour & McKerns, 1966) which is consistent with evidence that progesterone causes insulin insensitivity (Beck, 1969b; Costrini & Kalkhoff, 1971; Sutter-Dub et al., 1973). Reports concerning the effect of progesterone on hepatic glucose metabolism are inconclusive (Matute & Kalkhoff, 1973; Paul & Duttagupta, 1973).

A mild catabolic effect of oestrogens and progestins on the nonsexual organs in a number of species has been indicated, but this remains in doubt (Seal & Doe, 1969).

The involvement of neural mechanisms in the regulation of glucose homeostasis is poorly understood. That progesterone

increases appetite and reduces locomotor activity while oestrogens produce the opposite effects on these phenomena is generally recognized (Wade, 1972) but the concept of a central "glucostat" (Tepperman, 1968) or other neural principle through which sex steroids might influence glucose metabolism has yet to be characterized. Further studies are also required to confirm suggestions that sex steroids modify gastro-intestinal absorptive functions (Fisher, 1955; MacDonald & Crossley, 1970).

Thus it is generally implied that oestradiol and progesterone improve the ability of the female rat to regain normal blood glucose levels following a hyperglycaemic challenge. This is associated with an elevation of the circulating insulin level, and probably involves alterations of other endocrine phenomena, neural mechanisms and the general balance of intermediary metabolism. Oestradiol facilitates a hypoglycaemic action through effects on glucose uptake by the uterus and adipose tissue and it depresses hepatic glucose release. However, available evidence suggests that progesterone may antagonise the peripheral actions of insulin. Extensive further investigations are required to provide a greater understanding of these phenomena.

Reproductive States

On the basis of present knowledge of the effects of sex hormones on circulating insulin levels and blood glucose homeostasis a superficial view of the importance of these effects during different reproductive states in the female may be offered.

Pregnancy

The apparent diabetogenic stress of pregnancy is believed to reflect a rise in the circulating levels of insulin antagonists such as HPL and glucocorticoids. The raised levels of oestrogens and progestins during pregnancy may help to counteract this stress through their insulotrophic and hypoglycaemic tendencies. In diabetic pregnancy the circulating level of HPL is often excessive while the oestrogen titers are comparatively low. This combination may be implicit in the severe diabetogenic stress incurred by these patients.

Oestrous cycle

The markedly raised levels of oestrogens during mid and late pro-oestrus undoubtedly facilitate uterine development through the enhancement of glucose uptake by the metrial muscles. It is likely that the influence of sex steroids on carbohydrate metabolism during the cycle is intimately associated with behavioural features, particularly the reduction of feeding and the increase of running activity during oestrus.

Ageing

The reduced efficiency of the reproductive system in the ageing female rat, which indicates a decline of gonadal endocrine function may be a contributing factor in the age-related deterioration of glucose tolerance. This view is supported by the observation that long term ovariectomy accentuates the impairment of glucose tolerance in ageing rats. It is possible that analogous factors are involved in the aetiology of maturity-onset diabetes in women.

Male rat

Evidence to suggest a role of sex hormones in the regulation of blood glucose homeostasis in the male rat is derived from only a few isolated observations. Testosterone does not appear to influence blood sugar levels although it lowers plasma insulin levels and is generally held to promote the accumulation of muscle glycogen. One might therefore suggest that testosterone increases insulin sensitivity.

Since further data are not available it may be premature to speculate on mechanistic aspects. To date it is known that testosterone stimulates growth hormone secretion (Catt, 1971) and promotes feeding, exercise and protein anabolism (Seal & Doe, 1969); and studies in man indicate that testosterone has little effect on the level of blood lipids (Borenstein et al., 1973).

Blood sugar and plasma insulin levels are apparently unaltered by gonadotrophins; and melatonin, which inhibits insulin secretion in vitro does not produce a significant effect in vivo, even at a high concentration.

The importance of sex hormones in the regulation of blood glucose levels in the male therefore appears to be considerably less than in the female.

Sex differences

Characteristic differences of the shape of the intraperitoneal glucose tolerance curve in gonadectomized male and female rats, which may be modified but not eliminated

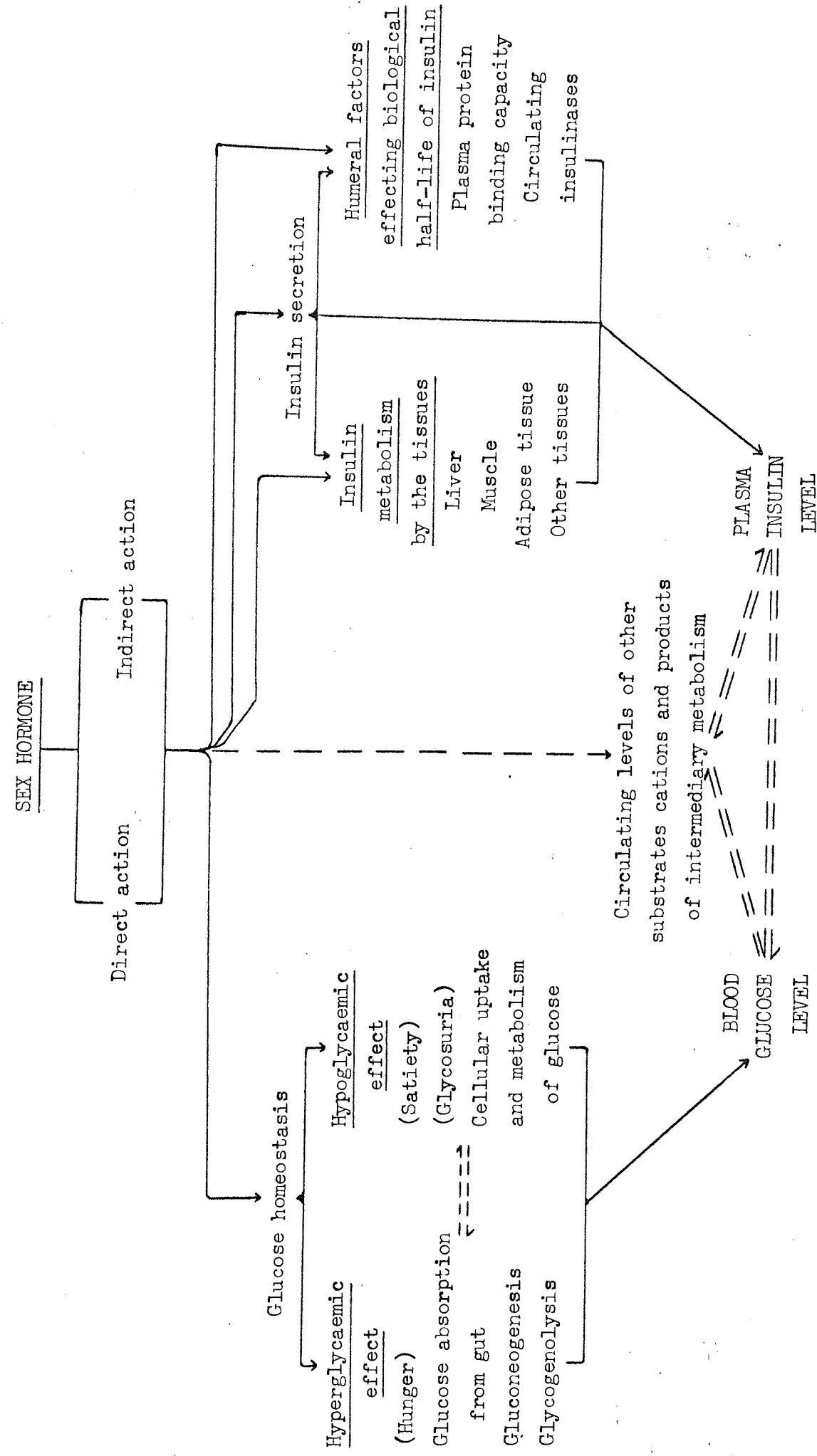
by gonadal integrity, indicate the existence of inherent sex differences of carbohydrate metabolism. This is further exemplified by the different effects of sex hormones in gonadectomized individuals of each sex and illustrates the differing roles of sex hormones in the regulation of carbohydrate metabolism in males and females. It is possible that sex differences observed in gonadectomized animals reflect, in part, an earlier exposure to a particular sex hormone environment. An appreciation of the nature of sex differences in the glucose homeostatic mechanism must await further elucidation of this phenomenon in each gender.

Concluding comment

The studies reported in this thesis have undoubtedly raised more questions than they have provided answers. Appropriately, this situation has been put into perspective by Claude Bernard. Speaking on the experimental approach to medicine, he said: "The only thing to do is lay the foundations upon which future generations may build, to create the physiology upon which this science may later be established." We continue to build.

Figure 25
Possible modes of action of sex hormones
on blood glucose and plasma insulin levels

FIGURE 25



APPENDIX

Appendix iTHE ISOLATED PERFUSED RAT PANCREASIntroduction

The isolated perfused pancreas provides an in vitro preparation for the study of insulin secretion under conditions which closely simulate the physiological state. For this reason it is generally deemed the most desirable method available for studying insulin secretion in vitro.

An apparatus for perfusion of the isolated rat pancreas was originally developed by Long (1946), and in spite of the relatively insensitive insulin bioassay method employed, it was clearly shown that glucose stimulates insulin secretion (Anderson & Long, 1947). Following the development of sensitive insulin radioimmunoassay methods Grodsky, Batts, Bennett, Ucella, McWilliams & Smith (1963) and Sussman, Vaughan & Timmer (1966) conducted further perfusion studies using systems essentially similar to the one described by Anderson & Long (1947). A characteristic feature of this type of system was re-circulation of the perfusion fluid. Several problems were encountered with this design. The composition of the perfusion fluid could not be maintained absolutely constant since metabolites accumulated and substrates were withdrawn. The exact dilution of test substances could only be determined by analysis of a sample of perfusate and it was difficult to determine the constitution of media required

to replace the fluid withdrawn for sampling. Particular attention centred around the supply of adequate oxygen to the isolated organ. Anderson & Long (1947) used a perfusate of heparinized whole blood which was re-oxygenated on each circuit by spreading into a thin film on the wall of a cyclindrical reservoir into which oxygen was blown. Whole blood was both expensive and difficult to circulate, and provided an intrinsic source of substances likely to modify insulin secretion. The later groups explored the introduction of artificial media but found that the oxygen carrying capacity of such media was not self-sufficient. Grodsky et al. (1973) used a 1:1 mixture of blood with 2% gelatin in saline and Sussman et al. (1966) used a 1:5 mixture of blood with 2.5% bovine albumin in Krebs bicarbonate ringer. Each system retained a complex oxygenation procedure based on the one described above.

Many of the problems relating to the re-cycling system were resolved with the introduction of a system in which perfusate was passed through the pancreas in a single passage (Curry, Bennett & Grodsky, 1968). This facilitated the use of an entirely artificial pre-oxygenated medium. The venous effluent could be collected continuously; the concentrations of test substances could be accurately determined without analysis, and the constancy of the perfusate could be maintained. Using this system Grodsky and his colleagues have contributed greatly to our appreciation of the kinetics of insulin release.

Several other groups of researchers are currently using a single passage rat pancreas perfusion system, and a system of this kind has been developed in the present study. It was envisaged that using this preparation the effects of sex steroids on insulin secretion in vitro could be investigated during a period of 6.5 hours. However, it was not possible to maintain the preparation for this period of time, and an alternative preparation (incubation of pancreas pieces) was used for this investigation. Nevertheless, in establishing the functional viability of the perfusion preparation several fundamental features of glucose induced insulin secretion were demonstrated.

Apparatus

The perfusion fluid was conveyed by a peristaltic pump (MHRE 88, Watson & Marlow Ltd., Falmouth) in a fine tube (polythene tube PP50, Portex Ltd., silicone tube 0.8 mm internal diameter, Watson & Marlow Ltd.) from the reservoir, through a pressure transducer (physiological pressure transducer, Bell & Howell Ltd., Basingstoke) to the tissue chamber. The pressure transducer was connected to a recorder unit (model M4, Devices Ltd., Welwyn Garden City, fitted with amplifier channels DC.2C and subunit 1,) to provide a continual monitor of perfusion pressure. Perfusate entered the tissue through the aortic cannula (PP100, Portex Ltd.) and was collected from the hepatic portal cannula (PP50) during serial one minute periods, using a fraction collector (type 7000, LKB, South Croydon). The tissue chamber was

thermostatically regulated at $37 \pm 0.1^{\circ}\text{C}$ and the reservoir was immersed in a waterbath at 40°C to compensate for the fall in temperature of the perfusate during its passage through the pump and pressure transducer. The dead space time was pre-adjusted at 30 seconds (varying not more than ± 1 second according to the pancreas circulation time of the individual preparation). All results were corrected for dead space. Control and test perfusates were prepared in separate reservoirs and introduced into the circuit by stopping the pump for approximately 1 second and manually transferring the capillary tube. The inner glass surface of the organ chamber was treated with Sigmacote. Figure 26 illustrates the complete perfusion system.

Surgical procedure for isolation and removal of the pancreas

The pancreas was isolated with the vasculature intact using a procedure based on that described by Sussman et al. (1966). Male Wistar rats weighing about 400 g were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (50 mg/kg body weight) and further doses of anaesthetic were administered as required. Individual steps in the operation are illustrated in plates C to G.

The abdomen was opened through a midline incision extending from the suprapubic region to the xiphisternum, and an incision at right-angles to this in the midlumber region. The first loop of the jejunum was cannulated between the first and upper second jejunal vessels, and transected immediately distal to the cannula. This cannula allows drainage of the exocrine

pancreas and the duodenum throughout the experiment, and prevents the development of a retrograde pressure in the pancreatic ducts. The colon was ligated and transected at the junction of the ascending and transverse portions, and the intestinal mesentery was ligated and transected between the jejunum and the ascending colon, permitting the removal from the abdomen of the jejunum, ileum, caecum and ascending colon together with their mesenteries. The omentum joining the transverse colon with the pancreas was removed by blunt dissection and the vascular connections between these tissues (the middle and right colic arteries and veins) were ligated and transected. The omentum connecting the descending colon to the dorsal wall of the abdomen was also severed by blunt dissection and the descending colon was ligated and transected posteriorly. The transverse and descending colons were then removed from the abdomen. The branches of the lienal artery and vein were ligated and transected distal to their emergence from the pancreas, and the spleen was removed. The oesophagus was ligated and transected as high as possible and the gastric branch of the coeliac axis was ligated and transected distal to the origin of the pancreatic artery. The omental connections between the pancreas and the greater curvature of the stomach were carefully severed by cauterization and the branches of the gastroepiploic artery and vein, which extend through the pancreas to the stomach were ligated and transected collectively in small groups. The anterior duodenum was cannulated through the pylorus and the stomach was removed.

The duodenal loop enclosing the "head" of the pancreas was retracted, and the right renal artery was cautiously exposed, ligated and transected between its origin and the point at which it passes beneath the inferior vena cava. The duodenal loop was returned and the "tail" of the pancreas was retracted. The abdominal aorta was carefully exposed from the crura of the diaphragm to below the left renal axis, and the inferior phrenic artery, superior suprarenal artery, left renal artery and left renal vein were separately ligated and transected. Connections between the aorta and the dorsal surface of the abdomen were sealed by cauterization and severed, and the aorta was ligated and transected between its point of emergence through the crura and the origin of the coeliac axis. The aortic cannula was introduced without delay into the section of aorta immediately distal to the left renal axis, thus leaving the superior mesenteric and coeliac axes intact. Perfusion was commenced at once so that the tissue was deprived of oxygen for only a few seconds. The aorta was transected below the aortic cannula, the pancreas was retracted to the right, and the venous cannula was inserted into the hepatic portal vein. The hepatic ligament was then ligated and transected, sealing the hepatic artery which emanates from the coeliac axis. The pancreas with the duodenum attached was dissected free of any remaining dorsal connections by cauterization, and lifted carefully into the tissue chamber of the perfusion apparatus.

The tissue was thus perfused via a retrograde aortic infusion into the coeliac and superior mesenteric vessels.

The former vessel subsequently supplies the pancreas through the pancreatic and superior pancreatico-duodenal arteries, and the latter through the inferior pancreatico-duodenal arteries, as illustrated in figure 27. Throughout the operation, which lasted about one and a quarter hours, the tissue was frequently moistened with warm Krebs bicarbonate ringer. An injection of heparin was not found necessary, since the prompt infusion of oxygenated perfusate was sufficient to flush the tissue before blood clotting could occur.

The venous perfusate was collected from the portal cannula over 1 minute periods at intervals indicated in the results. Each point refers to the concentration of insulin in the sample collected during the 1 minute preceding that point. 100 μ l aliquots of each sample were immediately withdrawn and stored at -20°C until analysed for insulin.

The tissue chamber was filled with a glucose-free solution of Krebs bicarbonate ringer (Zarrow et al., 1964) to which 4.5% polyvinylpyrrolidone (molecular weight approximately 44,000) was added as a colloid source. The perfusion fluid consisted of a similar solution to which glucose and test substances were added as required. Both fluids were equilibrated with $\text{O}_2 + \text{CO}_2$ (95:5), giving a pH of approximately 7.35. The perfusate was infused at a rate of 2.0 ml/minute and pressures remained steady within the range 20-70 mmHg.

Equilibration period

Experiments were conducted to establish the period of perfusion required for the pancreas to produce a steady response,

both in the absence of glucose and in the presence of 100 mg/100 ml glucose. As shown in figure 28a, under each of these conditions a steady release of insulin was produced within 10 minutes. For all subsequent experiments an equilibration period of 20 minutes was employed.

Insulin response to glucose stimulation.

Following an equilibration period in perfusate devoid of glucose, the introduction of perfusate containing 50mg/100 ml glucose did not produce any detectable alteration in the rate of insulin release (fig.28b).

Perfusate containing 100mg/100ml glucose produced a rapid transient rise in the rate of insulin release, reaching a peak within 3 minutes and declining to a level slightly above that observed originally (in the absence of glucose) (fig.28c).

Prolonged infusion with 100mg/100ml glucose during 20 minutes equilibration and 100 minutes test perfusion was associated with a steady release of insulin which started to decline towards the end of the test perfusion period (fig.28d).

After 20 minutes equilibration with 100mg/100ml glucose, the infusion of 300mg/100ml glucose produced a biphasic insulin response (fig.28e). Initially there was a rapid spike of release reaching a peak within 2 minutes and declining by 5 minutes. This was followed by a slowly generated increase in insulin release which continued to rise up to 55 minutes.

Histology

During selected experiments, portions of the splenic

region of the pancreas were removed prior to perfusion (usually at the time of gastrectomy) and at the end of the perfusion. Histological sections of the tissue were prepared and stained with H.E. or Gomori's A.F. Sections of the same pancreas at gastrectomy and following 80 minutes of perfusion. (20 minutes equilibration in perfusate containing 100mg/100ml glucose and 60 minutes test perfusion in perfusate containing 300mg/100ml glucose) are illustrated in plate H. After the perfusion there was evidence of tissue oedema, but there were no detectable abnormalities of the gross morphology of the islets or of the amounts of A.F. positive staining granular material.

Discussion.

On the basis of insulin secretory activity and histological evidence presented above, it appears that the preparation functioned normally for about 80 minutes. Beyond this time the secretory activity tended to decline and considerable oedema developed.

The insulin responses to the various glucose stimuli confirmed earlier observations by Grodsky and co-workers (Curry et al., 1968; Grodsky, 1973) and other researchers (Basabe, Lopez, Viktora & Wolff, 1971a,b; Landgraf, Kotler-Brajtburg & Matschinsky, 1971). Evidence of a similar nature has been observed during in vivo studies in man (Porte & Bagdade, 1970). Current knowledge from in vivo and from in vitro sources has led Porte & Bagdade (1970) and Grodsky (1973) to develop independently the concept of a

two-compartmental model for the release of insulin. Expanding the concept mathematically, Grodsky (1973) has suggested that the granular store of insulin within the beta cells is composed of a labile pool and a stable pool. The former is much smaller than the latter and responds immediately to certain thresholds of glucose by releasing specific quantities of insulin. Thus 50mg/100ml glucose is below the initial threshold level and does not produce an insulin response, while 100 and 300 mg/100ml glucose produce a rapid spike of insulin release related to the height of the stimulus. During prolonged glycaemic stimulation the stable pool releases insulin slowly, probably through the labile pool, and newly synthesized insulin replenishes the pools and increases this phase of insulin release. The two-compartmental concept of insulin release is gaining wide acceptance.

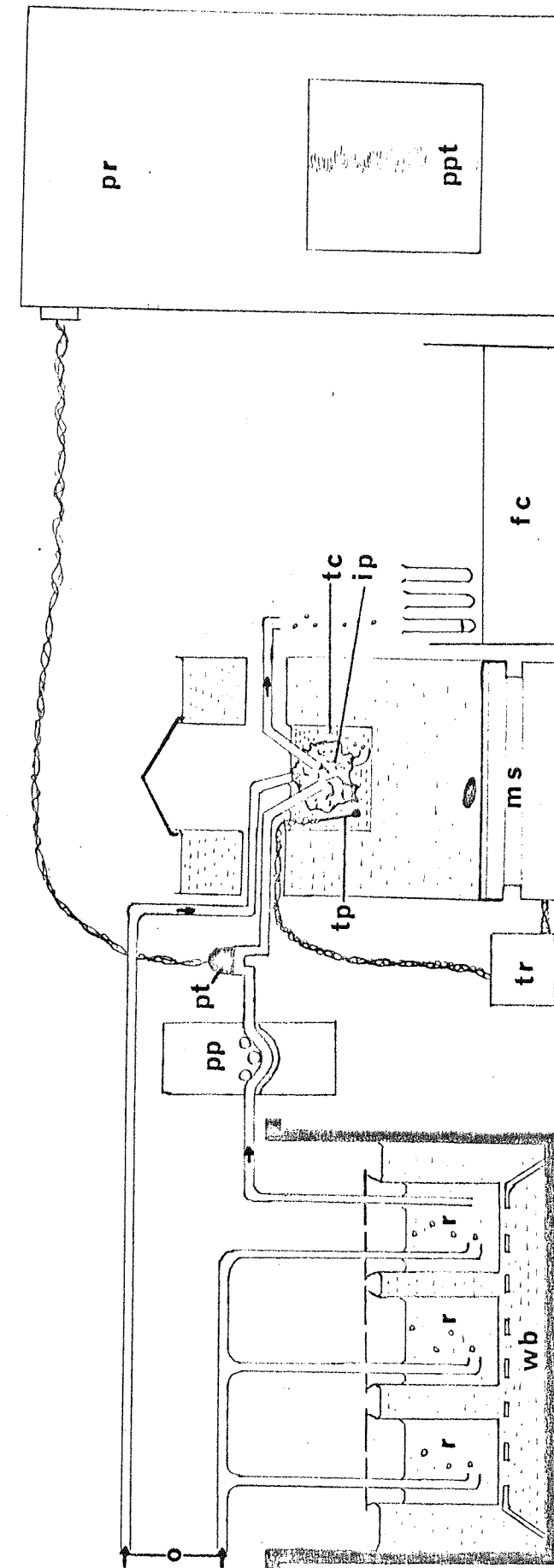
Several improvements of the present perfusion system were envisaged to enable experiments lasting several hours. However, the financial implications of these improvements rendered them impracticable. A perfusate composed largely of rat serum would improve the osmotic relations of the preparation. A specially dialysed extract would be required to remove the smaller molecules that are likely to influence insulin secretion. Continual replacement of the fluid in the tissue chamber might also be an advantage since it is possible that metabolites accumulate in this fluid.

Description of Figure 26

System for perfusion of the isolated rat pancreas

fc	fraction collector
ip	isolated pancreas
ms	magnetic stirrer-hot plate
o	$O_2 + CO_2$ (95:5)
pp	peristaltic pump
ppt	perfusion pressure tracing
pr	pressure recorder
pt	pressure transducer
r	reservoir containing oxygenated perfusate
tc	tissue chamber at $37^\circ C$
tp	thermistor probe
tr	temperature regulator
wb	water bath at $40^\circ C$

FIGURE 26



Description of Figure 27

Vasculature of the isolated perfused rat pancreas

ac	arterial cannula
ca	coeliac axis
d	duodenum
ga	gastric artery
gda	gastroduodenal artery
gea	gastroepiploic arteries
ha	hepatic artery
hpv	hepatic portal vein
ipa	inferior phrenic artery
ipda	inferior pancreatico-duodenal arteries
j1a	first jejunal artery
jl2a	lower second jejunal artery
ju2a	upper second jejunal artery
la	lienal artery
lra	left renal artery
mca	middle colic artery
pa	pancreatic artery
pla	pancreatic-lienal artery
rca	right colic artery
rra	right renal artery
sma	superior mesenteric artery
spda	superior pancreatico-duodenal arteries
ssa	superior suprarenal artery
tc	transverse colon
vc	venous cannula

FIGURE 27

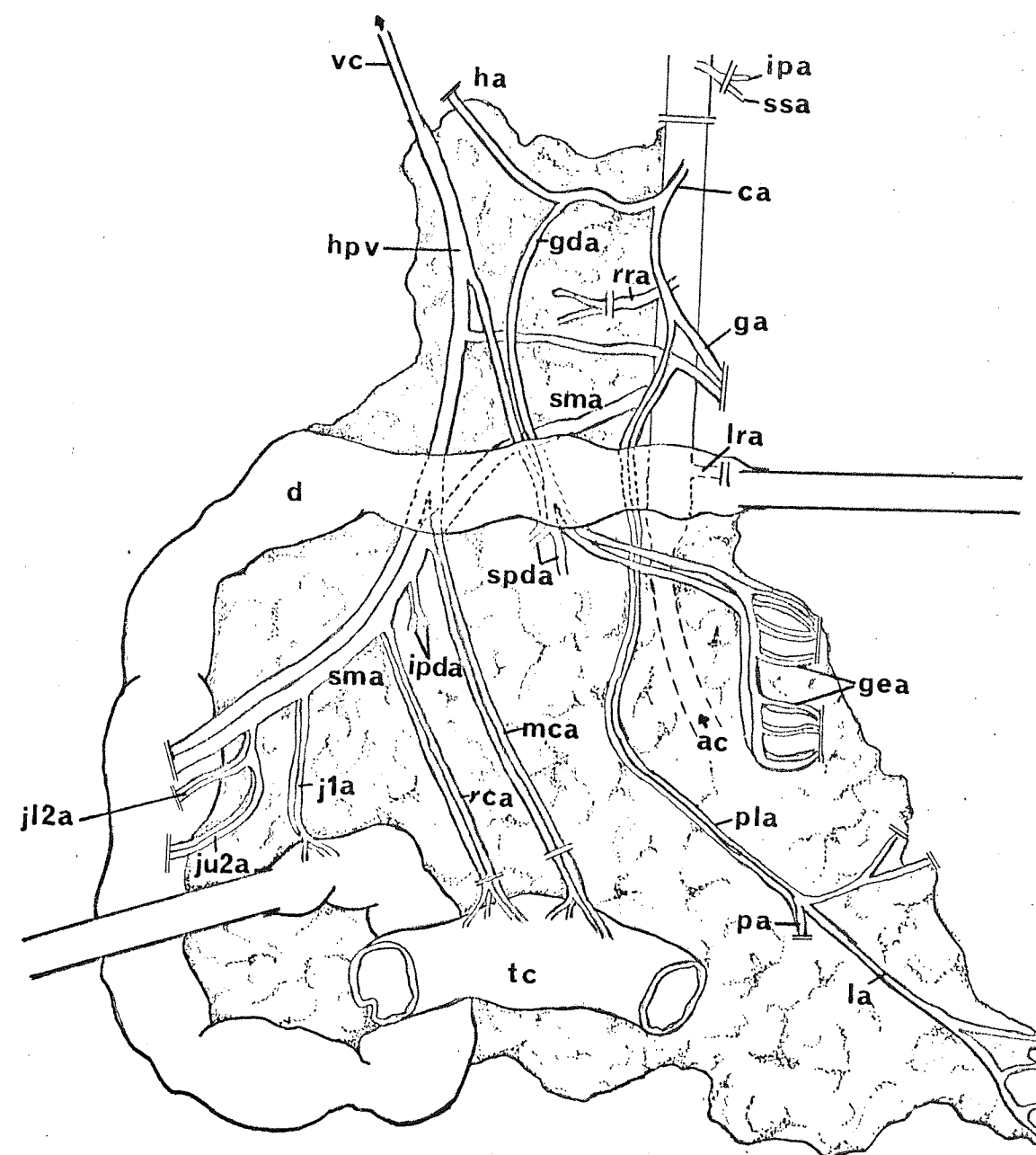


Figure 28 Kinetics of insulin release from the isolated perfused rat pancreas

Figure 28a Equilibration period for the production of a steady release of insulin

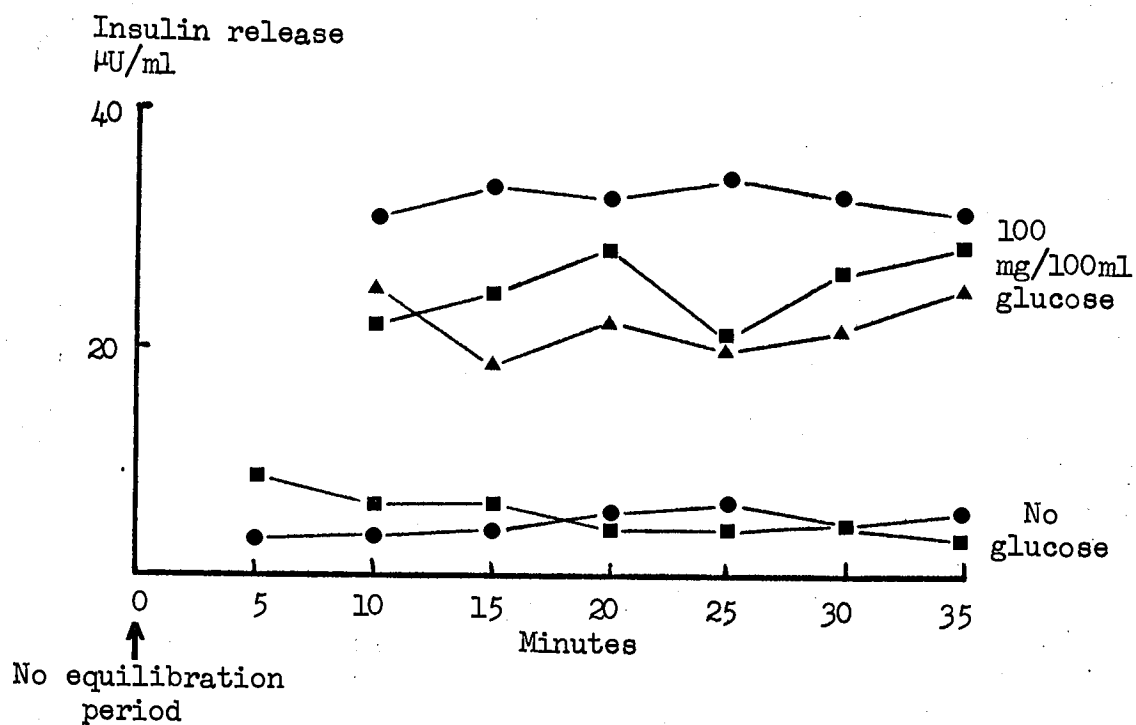


Figure 28b Insulin release in response to a constant infusion of 50 mg/100ml glucose

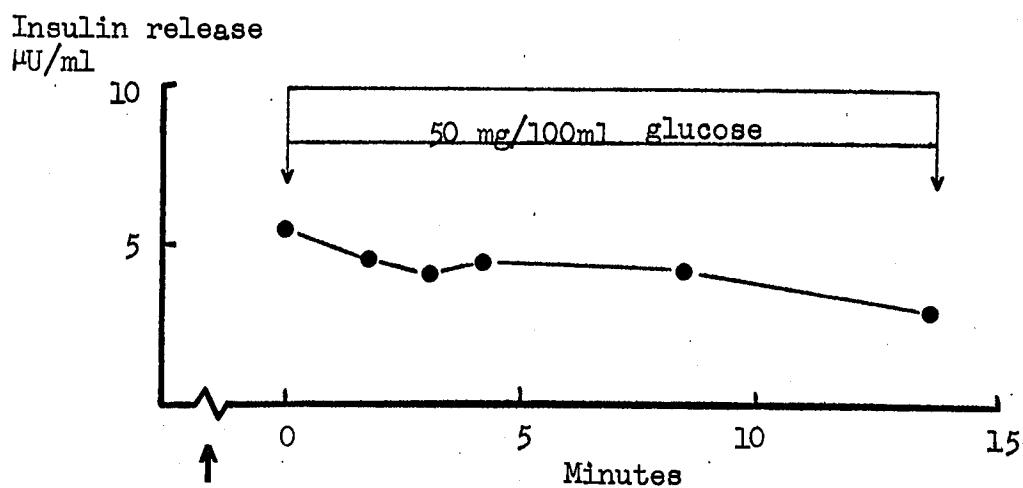


Figure 28c Insulin release in response to a constant infusion of 100 mg/100ml glucose

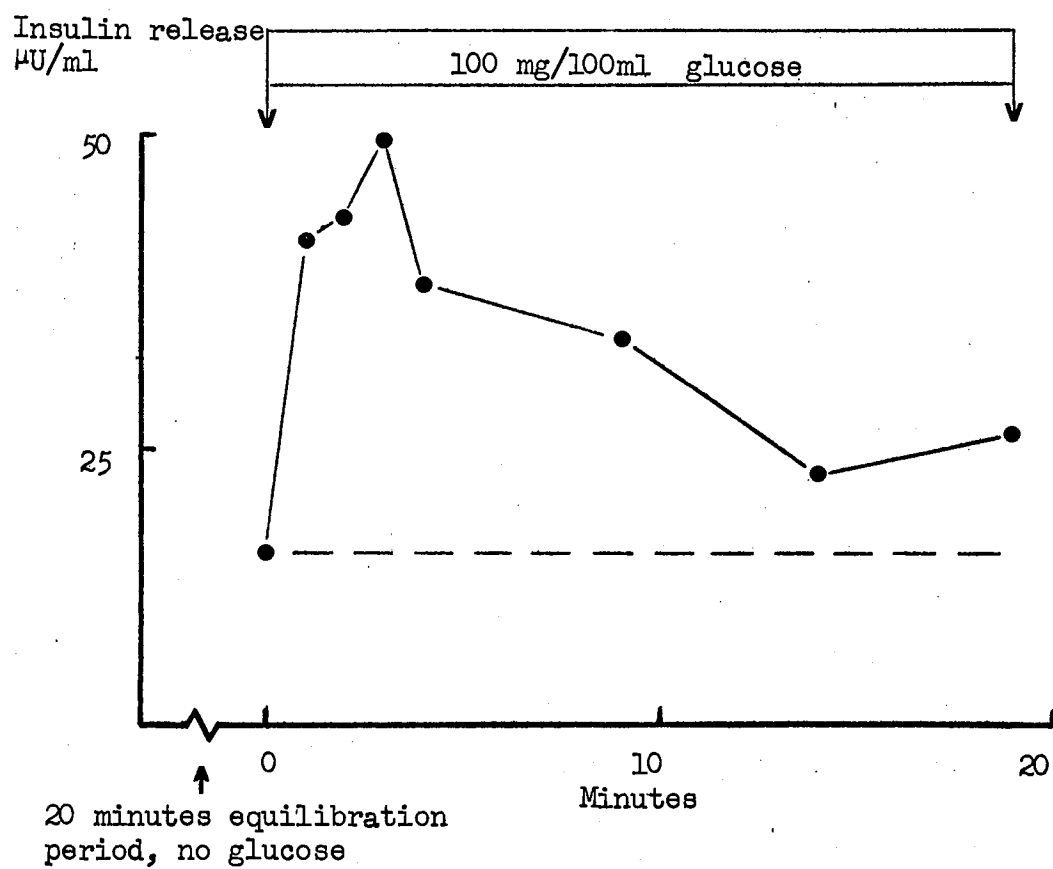


Figure 28d Insulin release during long term infusion of glucose, 100mg/100ml (mean \pm sem) (n = 3)

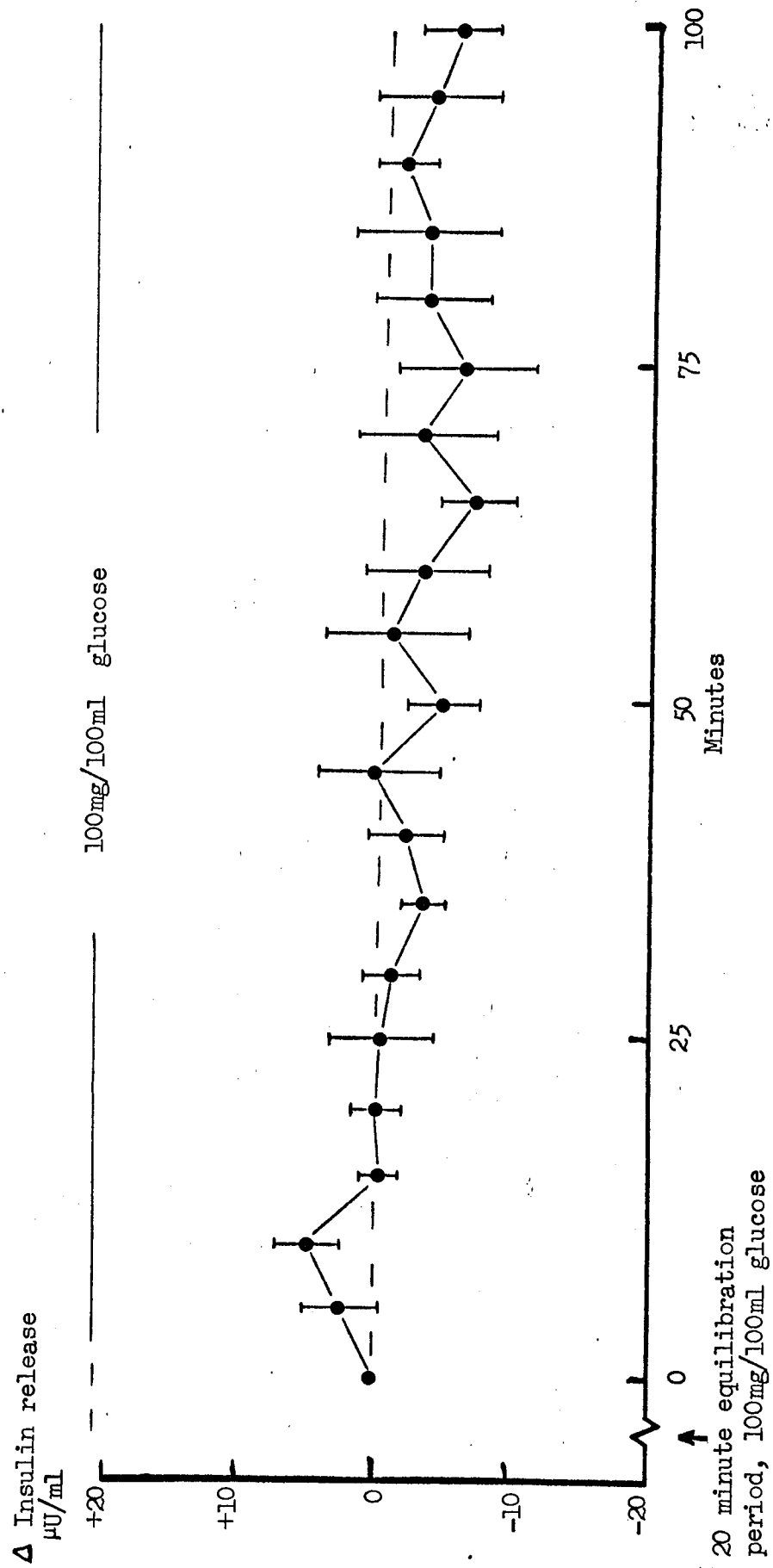
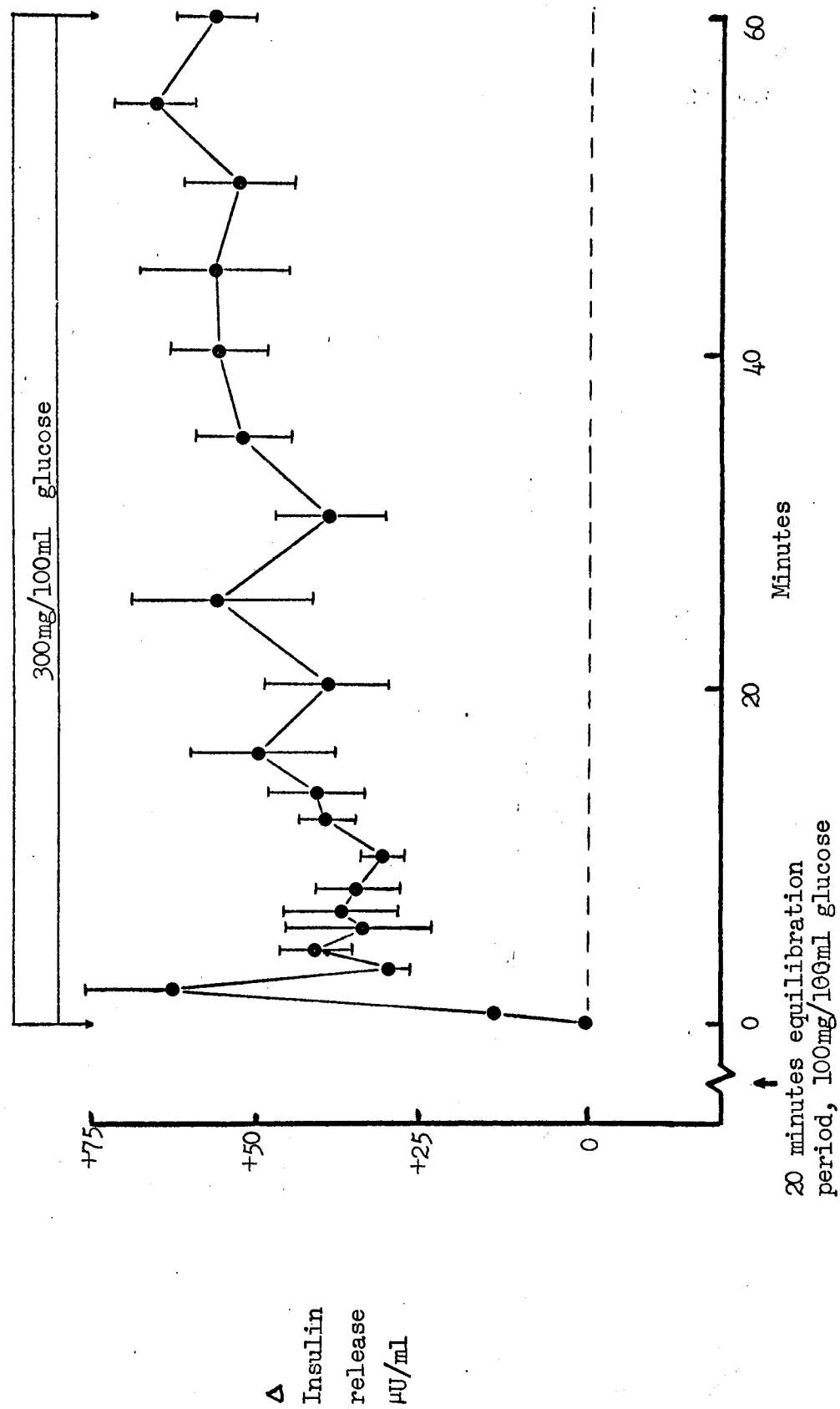


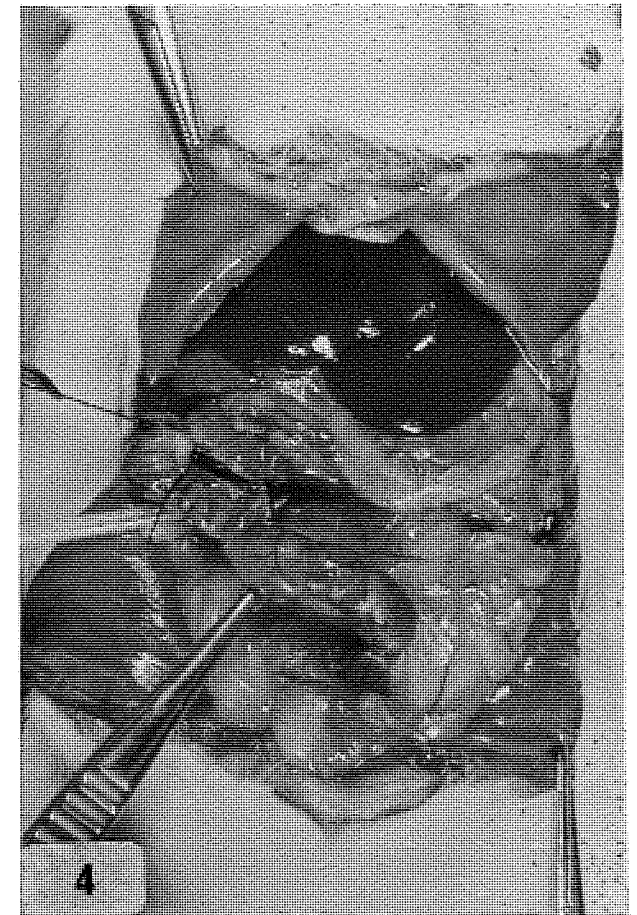
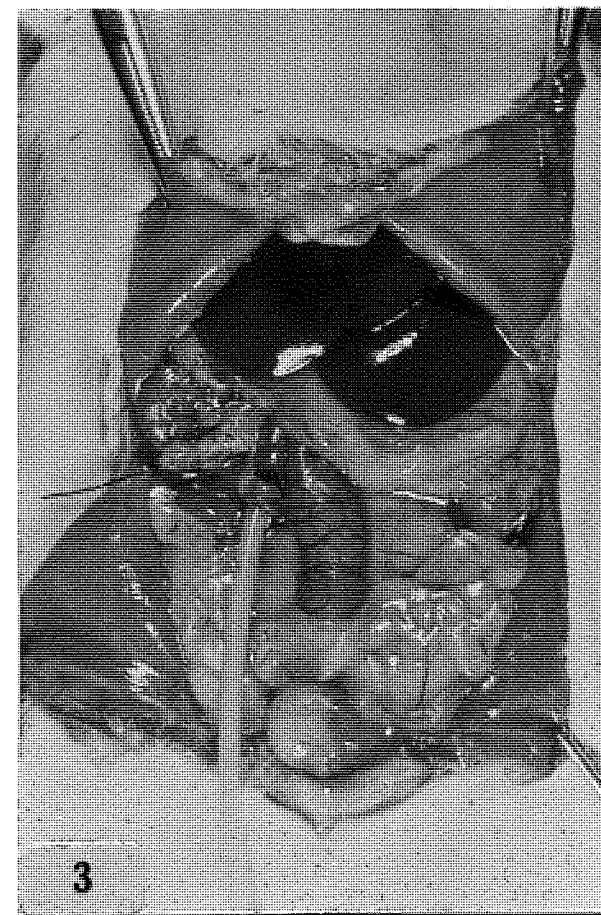
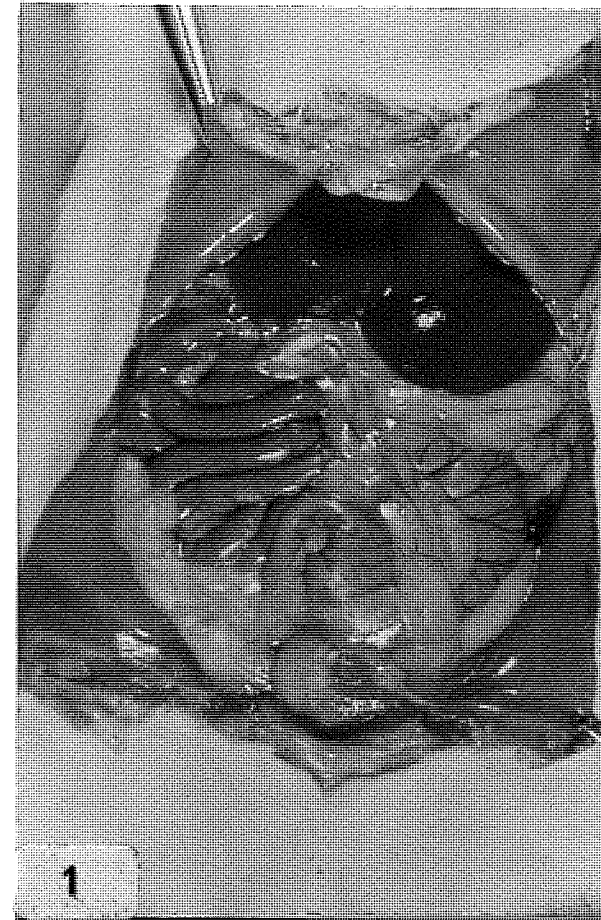
Figure 28e Insulin release in response to a constant infusion of glucose, 300mg/100ml
(mean \pm sem) (n = 3)



Description of Plate C

Procedure for isolation of the pancreas

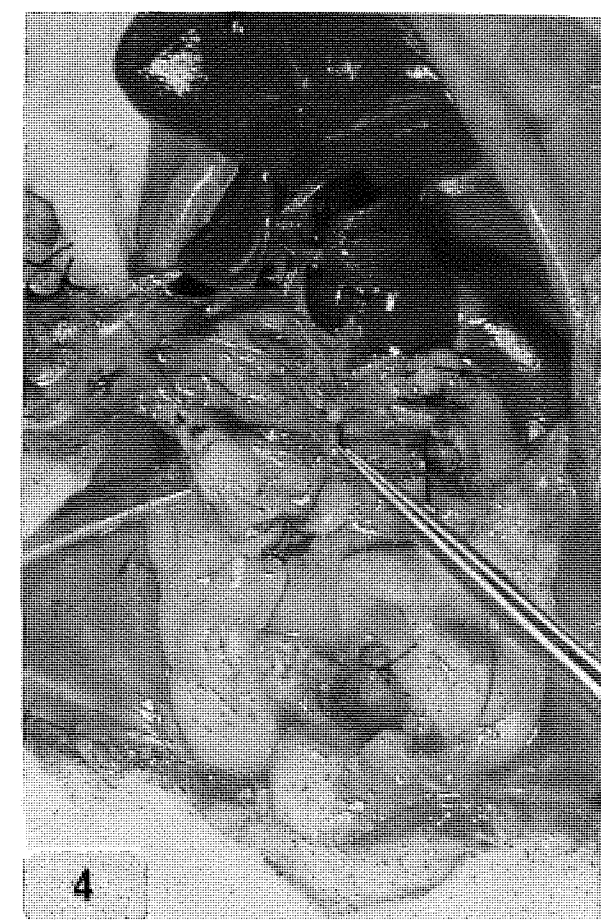
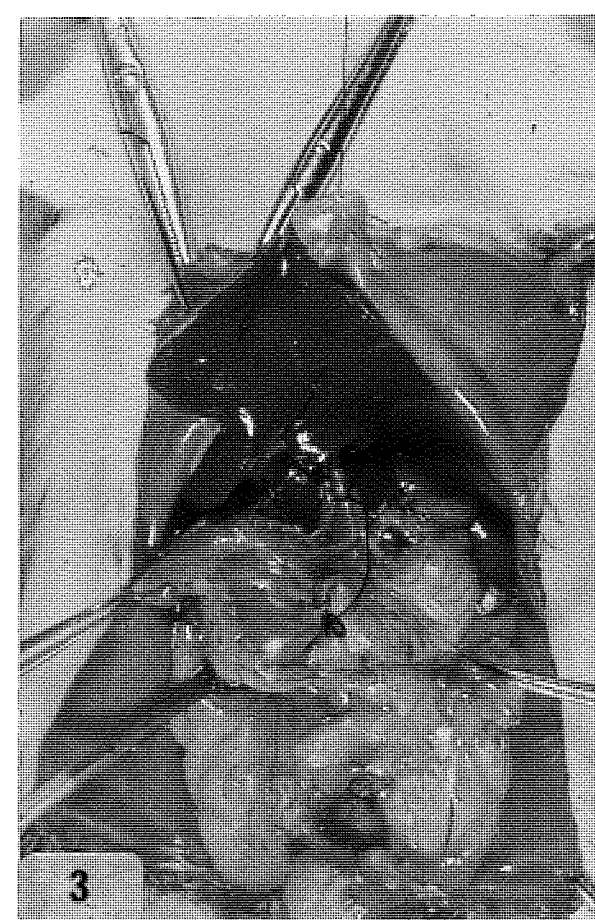
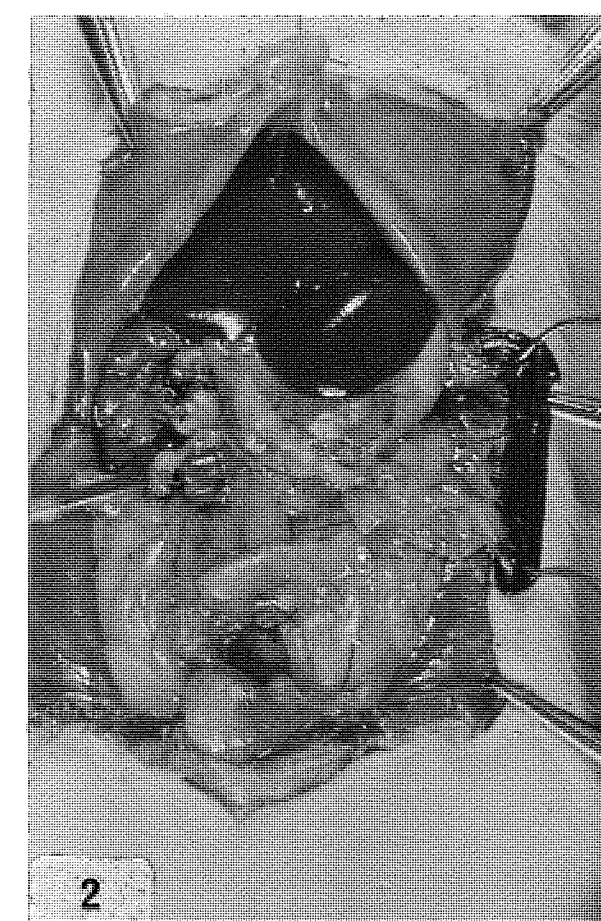
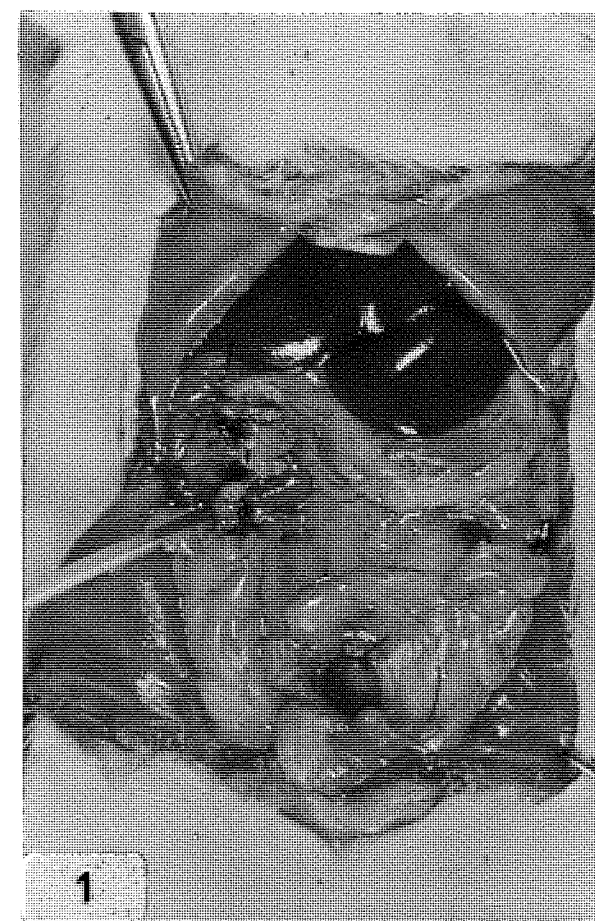
- 1 Display of abdominal contents.
- 2 Ligation of jejunum between first and upper second jejunal vessels.
- 3 Cannulation of jejunum; ligation and transection of junction of ascending and transverse colons; ligation and transection of mesentery of the small intestine, and removal of the ascending colon.
- 4 Removal of transverse and descending colons; ligation of vascular connections from pancreas to large bowel.



Description of Plate D

Procedure for isolation of the pancreas, continued

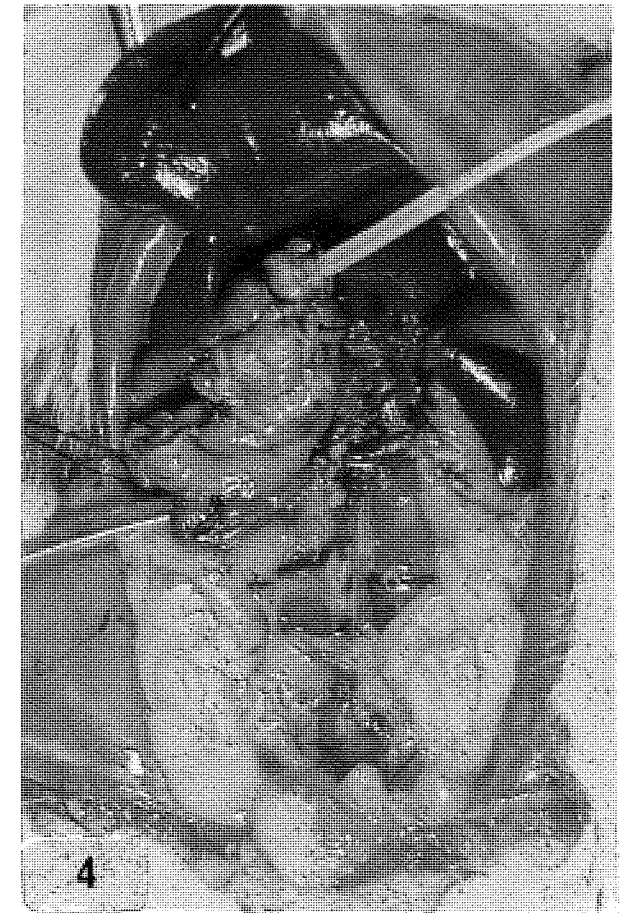
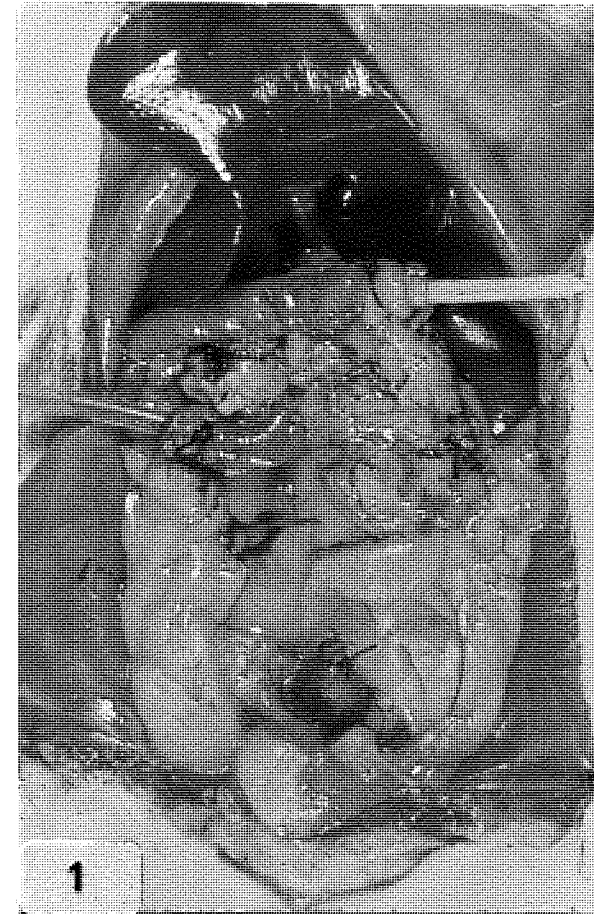
- 1 Transverse and descending colons removed.
- 2 Display of spleen and lienal vessels.
- 3 Lienal vessels ligated and transected; spleen removed; stomach retracted downwards, exposing oesophagus doubly ligated.
- 4 Display of coeliac artery and its branches; gastric branch severed (stomach for test)



Description of Plate E

Procedure for isolation of the pancreas, continued

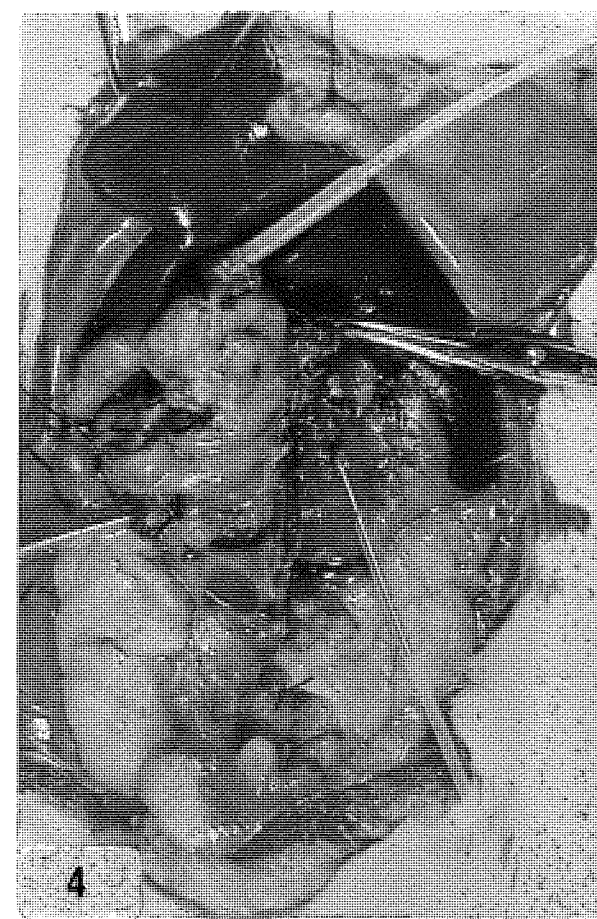
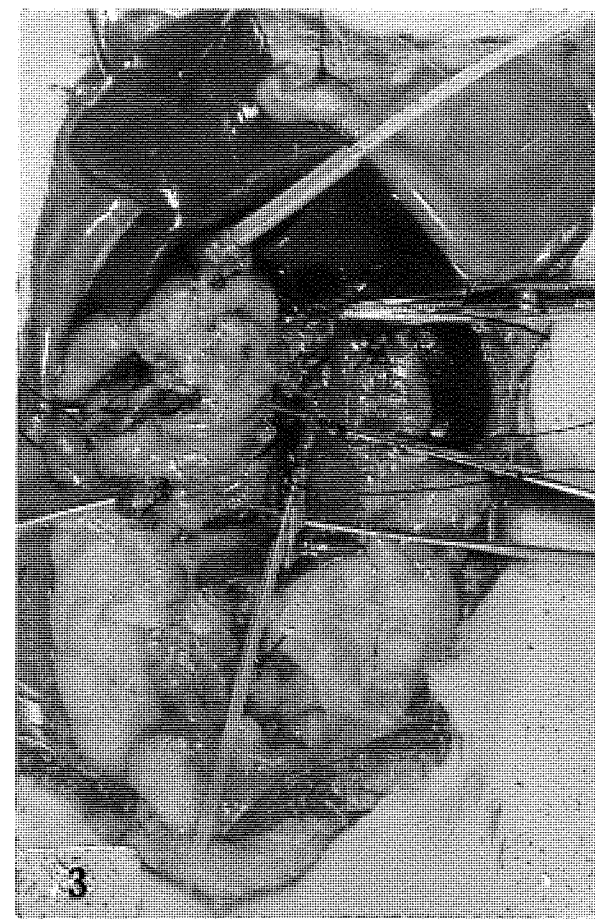
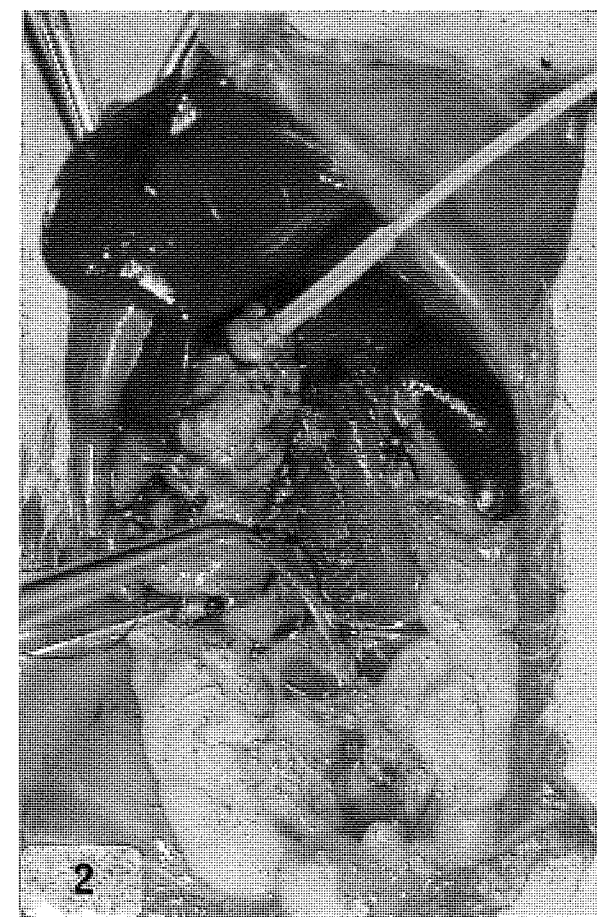
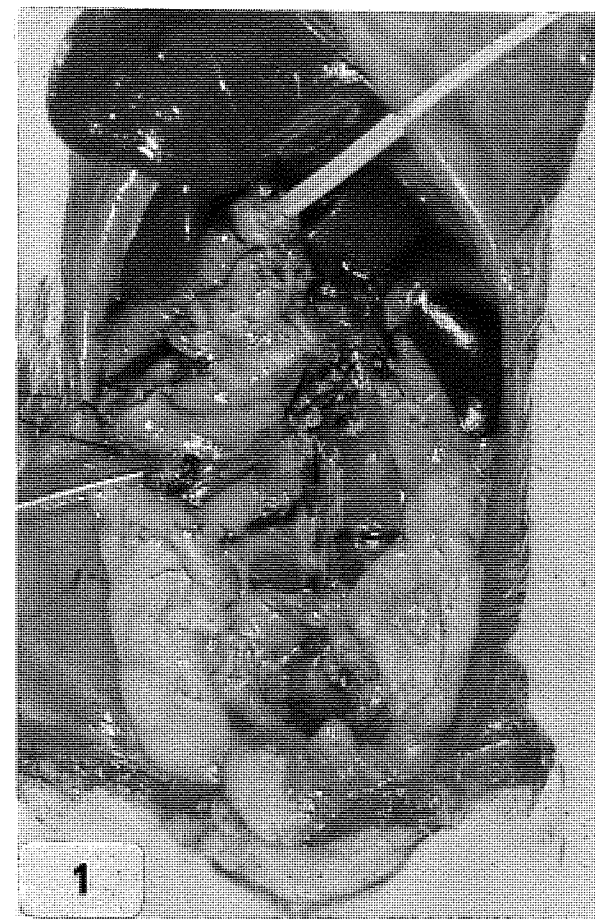
- 1 Cannulation of duodenum via pyloric sphincter; stomach removed.
- 2 Duodenal loop of pancreas retracted to display hepatic portal vein.
- 3 Double ligation and transection of right renal artery.
- 4 Tail of pancreas retracted over duodenal loop; left renal vein and left renal artery (hidden) are ligated close to the kidney; left renal vein is ligated at its junction with the vena cava; superior suprarenal and phrenic arteries are cauterized; suprarenal vein is ligated and severed.



Description of Plate F

Procedure for isolation of the pancreas, continued

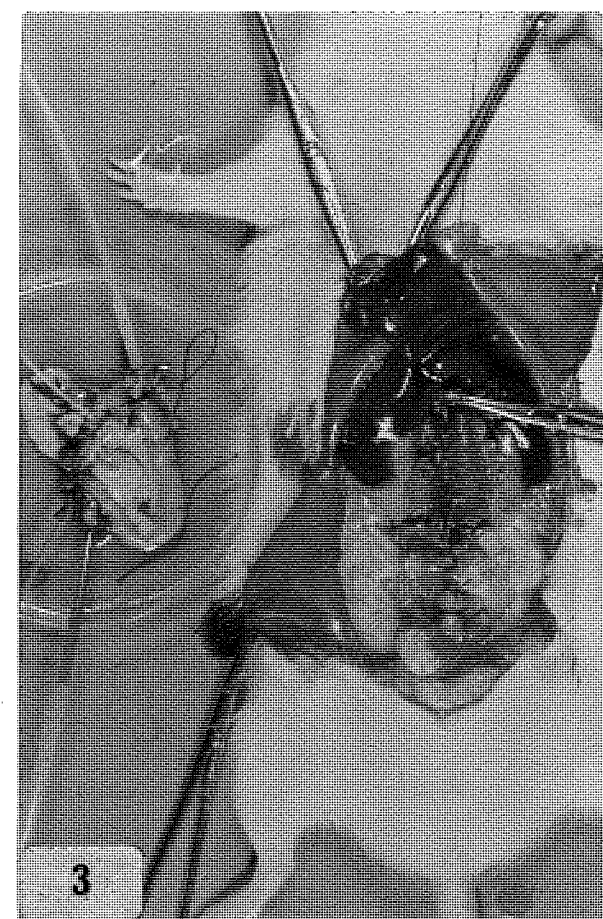
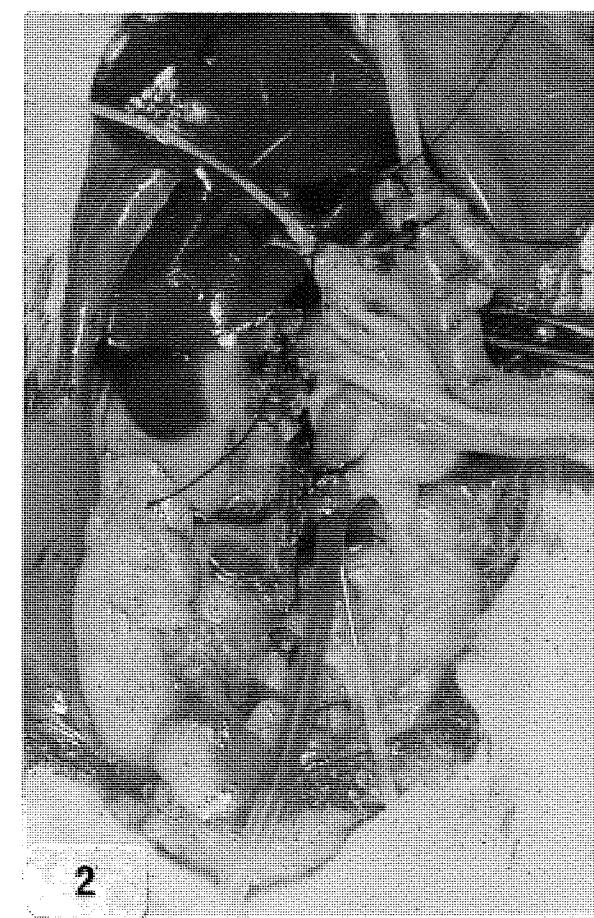
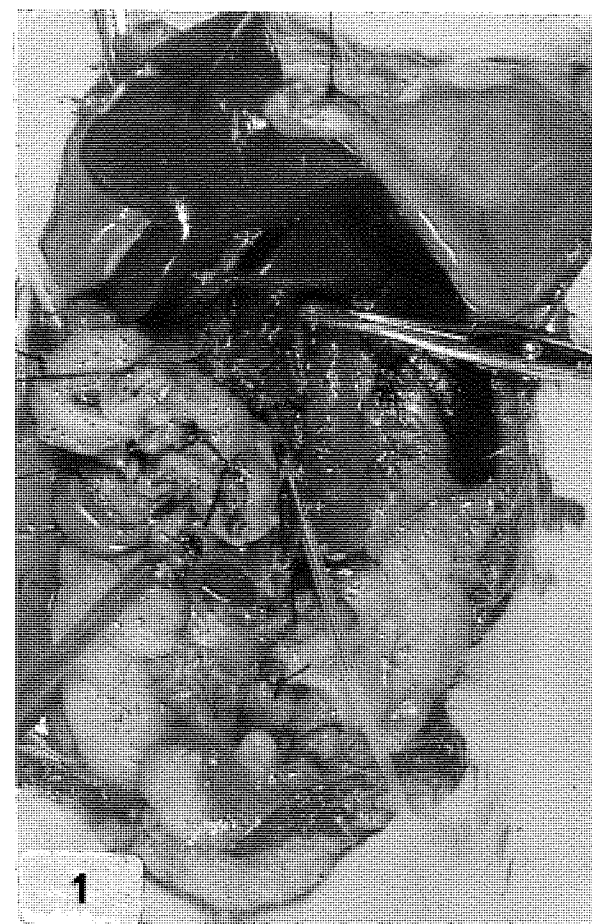
- 1 Left renal vein is transected; left renal artery is ligated close to aorta and transected.
- 2 Aorta is separated from the vena cava; dorsal vessels from the aorta are ligated (or cauterized) and transected.
- 3 Aorta is clamped and ligated above the point of emergence of the coeliac artery; the aortic cannula is inserted between the left renal artery and the iliolumbar arteries.
- 4 Aorta is ligated and transected below the cannula.



Description of Plate G

Procedure for isolation of the pancreas, continued

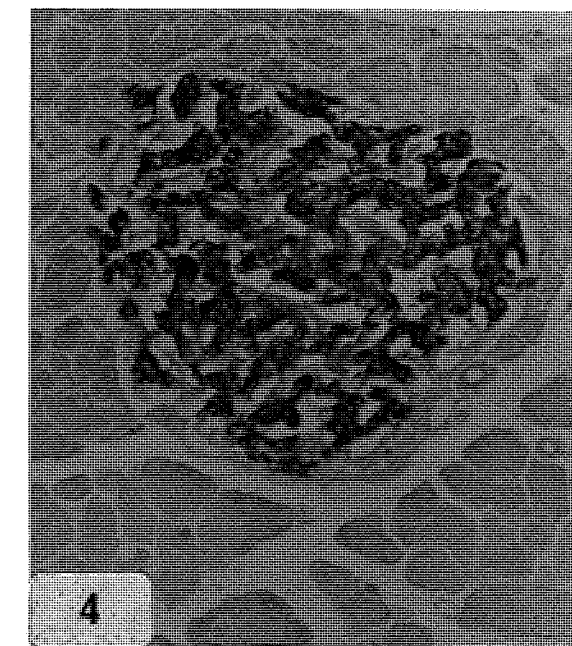
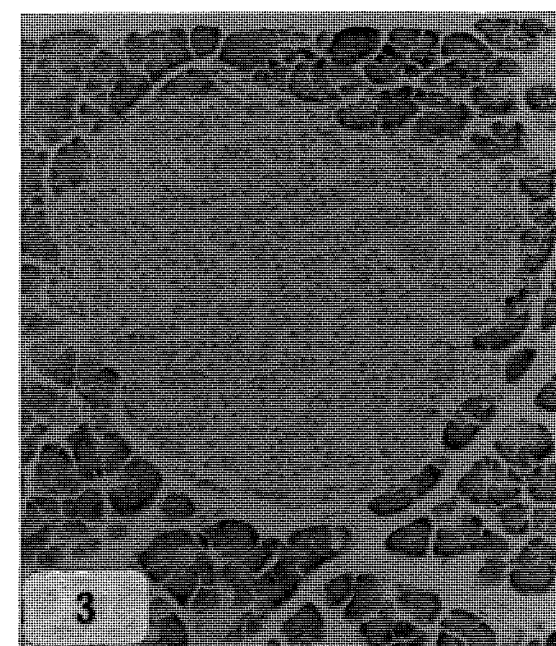
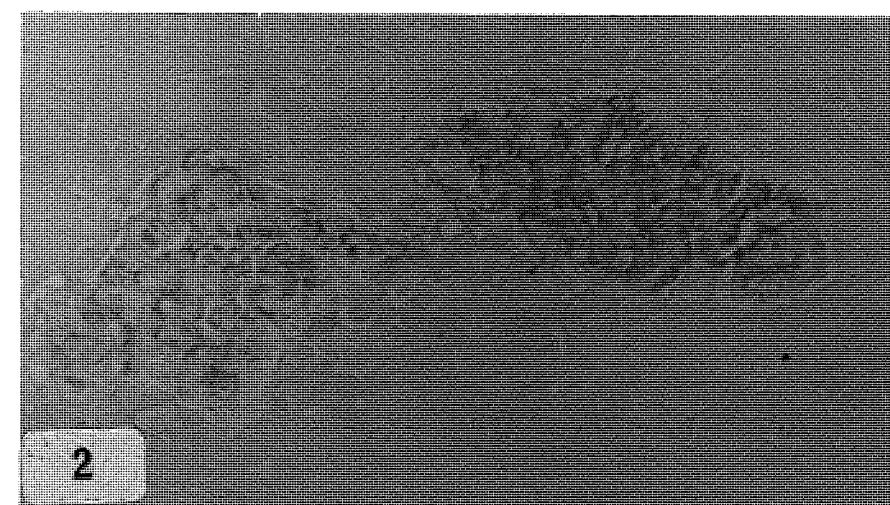
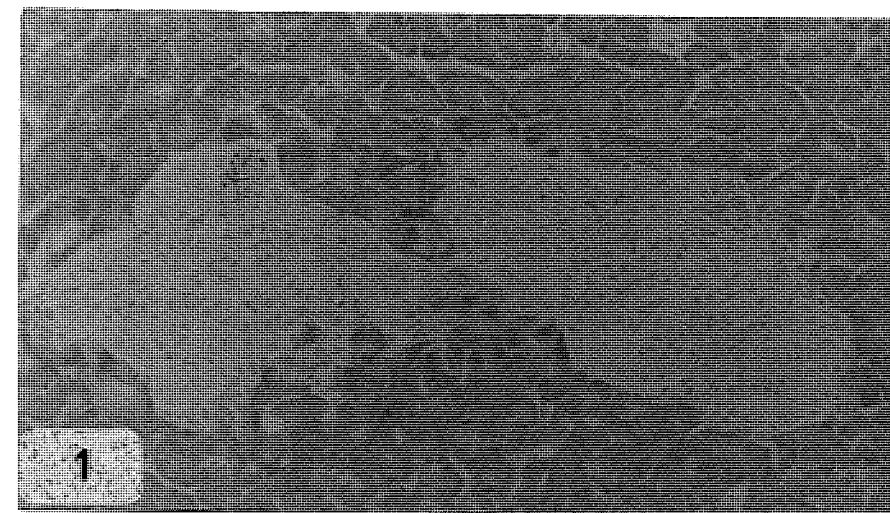
- 1 Aorta is transected above the point of emergence of the coeliac artery.
- 2 Duodenal loop of the pancreas is retracted; hepatic portal vein is cannulated (simultaneously ligating the hepatic artery and bile duct); the tissues become pale due to perfusion with colourless buffer.
- 3 Hepatic portal complex is transected; pancreas removed.



Description of Plate H

Islets of Langerhans from the same pancreas at gastrectomy and following 80 minutes of perfusion (20 minutes equilibration in perfusate containing 100 mg/100 ml glucose and 60 minutes test perfusion in perfusate containing 300 mg/100 ml glucose).

- 1 Islets at gastrectomy stained with H.E. (x 50).
- 2 Islets at gastrectomy stained with A.F. (x 50).
- 3 Islet after 80 minutes of perfusion stained with H.E. (x 50).
- 4 Islet after 80 minutes of perfusion stained with A.F. (x 50).



Appendix iiCONTRACEPTIVE STEROIDS AND CARBOHYDRATE METABOLISMIntroduction

It is under twenty years since the first clinical trials on steroid contraceptives were conducted (Rock, Pincus & Garcia, 1956) and already these drugs are being used by an estimated twenty million women throughout the world (International Planned Parenthood Federation, 1970).

Considerable concern has been aroused over possible hazards incurred both during and following their usage, and more than fifty different metabolic "side effects" have been indicated (see Salhanick, Kipnis & Vande Wiele, 1969; Briggs, Pitchford, Stamford, Barker & Taylor, 1970; Petrow, 1971; Haller, 1972; Warren, 1973).

In 1963, Waine and co-workers drew attention to an apparent diabetogenic influence of contraceptive steroids, and during the subsequent decade there have appeared more than one hundred publications on this topic, including several reviews (Spellacy, 1969a; Cohn, 1971; Kalkhoff, 1972; Beck, 1973). Most of the available evidence is derived from observations of glucose tolerance and plasma insulin levels. The present review incorporates a semiquantitative approach to this evidence and sets out to delineate the effects of contraceptive steroids, both individually and in the combinations commercially available, on these aspects of

carbohydrate metabolism.

The nature of contraceptive steroids

Contraceptive steroids are synthetic analogues of naturally occurring gonadal steroids. Their chemistry and modes of action as contraceptive agents have been reviewed on several occasions (Drill, 1966; Diczfalussy, 1968; Petrow, 1971; Haller, 1972), and only a brief resume is given below.

The convenience of an oral route of administration has heavily biased the selection of orally active compounds, but other routes of administration include pellet implants into the uterus, vagina, fat and muscle, and intramuscular depot injections. To increase the biological half-life an ethinyl or ester group is included at position 17, which renders the molecule more resistant to hepatic degradation.

Oral agents

Only three oestrogen analogues have been used for contraceptive purposes; ethinyl oestradiol, mestranol and quinestrol (table 5). Several kinds of progestins are in current use - mostly derivatives of either 19-nortestosterone or 17 α -hydroxyprogesterone. The former derivatives lack the 19 methyl group and exhibit powerful progestational effects; but they also possess mild androgenic activity (associated with the parent testosterone) and mild oestrogenic activity (both inherent and due to partial conversion to ethinyl oestradiol in vivo). 17 α -Hydroxyprogesterone is an intermediate in the biosynthesis of testosterone, and shows little biological activity: high progestational potency is achieved by esterification of the 17 α -hydroxyl group, and oestrogenic

and androgenic interference is negligible. A third kind of oral progestin with no oestrogenic or androgenic influences is derived from testosterone.

Implanted and injected agents

Many of the oral agents are administered in pellet form or as depot injections. Several further closely related compounds have been developed for these routes of administration (table 6).

Structural characteristics of these preparations are elaborated fully by Briggs et al. (1970) and Petrow (1971).

Treatment regimes and modes of action

The mode of action of contraceptive steroids is dependent on the treatment regime. Conception may be prevented through the inhibition of ovulation and/or through impaired sperm migration due to alterations of the cervical mucus and the contractility and glandular activity of the reproductive tract.

Combination pill. Oestrogen and progestin are administered for about 21 days of a 28 day cycle. The high steroid titers inhibit ovulation through suppression of the mid-cycle burst of LH.

Sequential pill. Oestrogen is taken alone, usually for 15 days, followed by oestrogen and progestin for 6 days of a 28 day cycle. The oestrogen inhibits ovulation through depression of FSH release, and the progestin facilitates withdrawal bleeding. The term 'serial' is frequently substituted for 'sequential' if a set of placebo tablets is included during menstruation.

Minipill. A daily low dose progestin is taken continuously. This does not appear to prevent ovulation, but it interferes with sperm migration.

Depot preparations. An oestrogen-progestin combination or progestin alone is administered as a pellet implant or an intramuscular injection. Such a preparation may be effective for several months. Oestrogen-progestin combinations inhibit ovulation while progestin alone impedes sperm migration.

Others. Recently, three other types of regime have been investigated: the pre-coital pill, a single low dose progestin which affords some contraceptive protection for about 18 hours; the weekend pill, a long acting combination pill that is taken once a week; and the post-coital pill, a short course of oestrogen therapy commencing soon after intercourse, which may produce an anti-implantation and/or an abortifacient effect.

A list of the treatment regimes of commercially available contraceptive preparations is given by Briggs et al.(1970).

Analysis of the literature

A number of problems were encountered in the construction of this review. Several authors have not provided full details about the subjects, contraceptives, investigative methods or the procedures used to analyse the results. Furthermore, mixed groups of data have been analysed together in certain publications, for example: non-diabetic subjects and subjects with various types of diabetes, subjects with different pathologies, and subjects receiving different contraceptive

preparations have been included in a single group. Factors such as these are likely to bear an important influence on the results obtained, and every attempt has been made to distinguish between them. Inevitably there are many gaps and question marks.

For reasons of clarity and brevity the substance of this review is presented largely in tabular form.

Changes of glucose tolerance and the circulating insulin response in groups of women receiving contraceptive steroids, as judged by comparison with "control" individuals, have each been ranked on an arbitrary scale from 'a' to 'd' according to the criteria defined in table 7. Thus, a particular group of subjects may have shown no change in glucose tolerance ('b') but a significant increase in the circulating insulin response ('d'); and in a series of studies the percentage occurrence of individual points on the scale may be determined. Using this system the results of many studies may be concisely abstracted and analysed.

It has been convenient to categorise the literature according to the type of contraceptive regime employed and the medical history of the subjects. The effects of combined, sequential, minipill and depot preparations have been evaluated in apparently normal healthy women, diabetic women, and women with other pathological conditions. In certain cases data relating to a single preparation or constituent compound have been examined. The majority of studies relate to women of child-bearing age, but since contraceptive steroids have also

been prescribed to alleviate symptoms of the climacterium, differences between pre- and post-menopausal women have been noted.

Criticisms of analysis procedure

An important criticism of this approach is the heterogeneity of the groups. To take into account most of the available evidence it was necessary to include within a single category studies on women of varying age, parity, duration of therapy and socio-ecological environment. Under the heading of 'apparently normal healthy women' there are studies in which some potential, latent and gestational diabetics and some obese subjects have not been distinguished from women without these conditions. The term 'diabetic' incorporates studies on latent, gestational, asymptomatic and clinical diabetic patients, and 'other pathologies' comprise patients with acromegaly, rheumatoid arthritis and certain complications of the uterus.

Pretreatment control and type of glucose tolerance test

Some investigators have estimated the effects of contraceptive steroids by reference to the same women before the beginning of treatment (pretreatment control), but others have compared women undergoing treatment with a separate, though similar group of women which is not receiving treatment. Glucose tolerance has been determined in some instances by oral tests and in other instances by intravenous tests, or both.

Extended analysis of studies with Conovid and Metrulen

preparations in apparently normal healthy women of child-bearing

age has shown that the results obtained do not correlate with the use of either a pretreatment control, or an oral on an intravenous glucose tolerance test (table 8).

Artificial menstrual cycles

Four studies have been concerned with possible variations of glucose metabolism during the artificial menstrual cycles of women receiving contraceptive steroids (table 9). Taylor & Kass (1968) were unable to identify any differences of glucose tolerance between day 15 and day 28 in women receiving the combination preparation Conovid; and Larsson-Cohn, Tengström & Wide (1969) found no evidence of differences in glucose tolerance or plasma insulin levels between the follicular and the luteal phase in women treated with two minipill progestins. An examination of women treated with a sequential regime, Serial 28, revealed that glucose tolerance was significantly impaired on days 21 and 22 in comparison with days 27 and 28 (Pyorala, Pyorala & Lampinen, 1967); and Foss, Holton & Lewis (1970) observed a tendency for glucose tolerance to decline as the cycle advanced in women treated with the minipill Norgestrel. Thus the available evidence is insufficient to conclude any consistent alterations of glucose metabolism during artificial menstrual cycles. A similar situation exists with regard to glucose metabolism during natural menstrual cycles.

Some of the studies listed below have defined the time of the cycle at which glucose tolerance tests were performed, but many have not taken this possible variable into account and only those mentioned above have actually investigated this aspect.

Alterations of carbohydrate metabolism during artificial menstrual cycles might contribute towards an explanation of some of the apparently paradoxical results that have emerged.

Discussion

Apparently normal healthy women

A striking feature of the literature (table 10, sections 1-4, 11 & 12) is the extensive spread of results with regard both to a particular regime and to an individual preparation. Some possible reasons for these inconsistencies have already been considered; others will be added below.

A comparison of the effects of different contraceptive regimes on glucose tolerance and the circulating insulin response in apparently normal healthy women of child-bearing age is shown in table 11.

Reference to the percentage occurrence of the grades 'a', 'b', 'c' and 'd' indicates that combined preparations produced an impairment of glucose tolerance and an elevation of insulin levels in the majority of studies. The incidence of impairment was lower for sequential preparations although elevated insulin levels were equally common. Minipill progestin therapy has been observed to impair glucose tolerance on fewer occasions than above, but there are insufficient data to determine a trend of changes in the insulin levels. Evidence relating to depot progestin treatment is also inadequate to identify any definite trends of either glucose tolerance or insulin levels. Thus it appears that glucose tolerance is generally impaired by contraceptive steroid preparations, particularly by combined

regimes and less frequently by other regimes.

Individual preparations undoubtedly produce different sorts of effects, but the many variables that are inherent in each investigation make it impossible for the nature of these differences to be satisfactorily distinguished by a survey of the literature. Several authors have directly compared glucose tolerance in similar groups of women receiving a combined or a sequential contraceptive (table 12), and in certain cases it was observed that the combined preparation produced a greater impairment than the sequential preparation. The majority of these comparisons, however, have not revealed a significant difference between the effects of these two regimes.

There are a number of reports to indicate that following the cessation of contraceptive steroid treatment glucose tolerance quickly reverts to the pretreatment condition (usually within a month), although the circulating insulin level may take longer to re-adjust (Halling, Michals & Paulsen, 1967; Starup, Dale & Deckert, 1968; Javier, Gershberg & Hulse, 1968; Goldman, Eckerling & Ovadia, 1969; Goldman & Eckerling, 1970b; Kalkhoff, 1972). Other sources indicate that glucose tolerance is dependent on the duration of treatment. They show that glucose tolerance is impaired to a greater extent during the initial months of treatment than after a year or longer (Javier et al., 1968; Posner et al., 1967a,b; Rice-Wray, DeFerrier, Perez-Huerta & Gorodovsky, 1970; Pyorala, Pyorala, Lampinen & Taskinen, 1971). It is possible that this variable might also contribute towards an explanation of the sometimes

apparently inconsistent reports.

The incidence of impaired glucose tolerance appears to be exaggerated among potential diabetics (Gershberg, Javier & Hulse, 1964; Posner et al., 1967a,b; Javier et al., 1968; Spellacy et al., 1968a,b; 1969a; 1971c,d; Goldman & Ovadia, 1969), and also among older women and obese women (Frerichs et al., 1966; Spellacy et al., 1968a; 1971a,c,d; 1972a; Phillips & Duffy, 1973). In a recent survey of the literature, Kalkhoff (1972) noted that contraceptive steroids rarely impaired glucose metabolism to the extent of diabetes if there are no indications of abnormal glucose tolerance prior to treatment.

The limited information regarding post-menopausal subjects comprises reports that combined and sequential preparations produce little alteration or an impairment of glucose tolerance (table 10, sections 11 & 12). Insulin levels were not affected in three out of four studies, but Pyorala, Pyorala, Lampinen & Taskinen (1970) observed a depletion of this hormone in women treated with a sequential drug for about a year.

Diabetic women

Changes of glucose metabolism observed in pre- and post-menopausal diabetic women receiving contraceptive steroids are summarised in table 10, Sections 5-8, 13 & 14.

In pre-menopausal gestational diabetics combined and sequential preparations generally impair glucose tolerance and elevate insulin levels as observed in normal healthy women; and in a single study on latent diabetics glucose tolerance was

impaired by a combined preparation.

There are reports that contraceptive preparations improve, impair and produce no alterations of glucose tolerance in asymptomatic and clinical diabetics of child-bearing age and older. The circulating insulin levels of these patients may also be raised, lowered or unchanged. These reports are generally consistent with the view that patients with an adequate insulin reserve may show some improvement of glucose tolerance as a result of the elevated levels of insulin in the blood, whereas patients with a compromised reserve show a deterioration of glucose tolerance due to their inadaptability.

A large proportion of asymptomatic diabetics with compromised insulin reserve develop clinical diabetes during contraceptive steroid therapy, and there is evidence that such therapy aggravates the diabetic condition (Spellacy, 1969; Kalkhoff, 1972). For these reasons contraceptive steroids are not recommended for prescription to asymptomatic or clinical diabetics.

Other pathologies

Isolated observations of the effects of contraceptive steroids on glucose metabolism in women suffering from various pathologies other than diabetes are listed in table 10, sections 9, 10 and 15. Depot progestins did not produce any changes of glucose tolerance in pre- and post-menopausal women with dysfunctional uterine bleeding or in pre-menopausal acromegalics, but there was evidence of an impairment among rheumatoid arthritics treated with the combination drug Conovid.

Synthetic oestrogens

Since the incidence of impaired glucose tolerance is much greater in women receiving oestrogen-progestin contraceptives than in women receiving progestin alone it is not unreasonable to suppose that synthetic oestrogens exert a diabetogenic influence. Spellacy, Buhi & Birk (1972c) evaluated the effects of ethinyl oestradiol and mestranol in non-diabetic women and concluded that these compounds do not produce any significant alterations of glucose tolerance or circulating insulin levels. However, it was noted that glucose tolerance became "improved slightly in the pre-menopausal women and deteriorated slightly in the post-menopausal women." These findings are not in line with earlier indications that mestranol impairs glucose metabolism in non-diabetic pre-menopausal subjects (Javier et al., 1968) and improves glucose metabolism in maturity-onset diabetes (Gershberg, Javier, Hulse & Cohane, 1967).

Other reports on this aspect are equally equivocal. The synthetic oestrogen stilboestrol and its diethyl derivative appear to impair glucose tolerance in healthy pre-menopausal women (Buckler & Warren, 1966). These oestrogens impair glucose metabolism in acromegalics (McCullugh, Beck & Schaffenberg, 1955) although this effect may be preceded by a transient improvement (Mintz, Finster & Josimovich, 1967).

Thus, studies on the oestrogenic components of contraceptive steroids are inconclusive, but the different effects of oestrogenic and non-oestrogenic preparations have provided a basis for the general belief that synthetic oestrogens are

potentially diabetogenic in younger women (Davidson & Holzman, 1973) although their effects are seldom marked and may well be obscured by contra-adjustments. These effects of synthetic oestrogens are in contrast to the effects of their naturally occurring counterparts.

Synthetic sex hormones in sub-human species

Experimental animals have been used on several occasions to investigate the interaction of contraceptive steroids and carbohydrate metabolism, but these investigations have added little to our overall comprehension of this area.

Female rats treated with five different oestrogen-progestin combined preparations showed no significant alterations of glucose tolerance in a study by Fenichel, Purse, Alburn & Edgren (1969), but one of these drugs, Conovid, produced a significant diabetogenic effect in a similar study by Lei & Young (1972). The latter workers also noted that Conovid impaired insulin sensitivity.

Rhesus monkeys treated with ethinyl oestradiol showed no apparent changes of glucose tolerance or plasma insulin levels, although there was evidence of impaired insulin sensitivity (Beck, 1969a). The administration of mestranol, however, elevated plasma insulin levels and impaired insulin sensitivity without altering glucose tolerance.

Several synthetic progestins have been shown to promote insulin secretion without altering glucose tolerance in monkeys, rats and dogs (Beck, 1969a; Goberna, Voigt, Fussganger, Laube & Pfeiffer, 1971; Orsetti & Bourgeois, 1971), supporting the

view that progestins impair insulin sensitivity. Chlormadinone acetate appears to exert a sex-dependent influence on the diabetic condition of partially pancreatectomized rats, improving the condition of females but exacerbating the condition of males; and these influences are abolished by gonadectomy (Fussganger, Goberna, Voigt, Laube & Pfeiffer, 1971; Goberna et al., 1971). These observations are in contrast to the work of Lewis et al. (1950) who reported that natural progestins do not effect the course of experimental diabetes.

Diethylstilboestrol ameliorates diabetes in partially pancreatectomized, gonadectomized rats in a similar manner to natural oestrogens (Rodriguez, 1965), but the activity of this compound also appears to be dependent on gonadal integrity, since Ingle (1941; 1943) observed that it aggravates the diabetes in sexually competent individuals. There is evidence that diethylstilboestrol, like natural oestrogens, promotes islet growth (Kerr, Stears, MacDougall & Haist, 1952) and hepatic glycogen deposition (Griffiths, Marks & Young, 1941).

Mechanisms of action

The possible mechanisms through which contraceptive steroids might effect glucose tolerance and circulating insulin levels are likely to include those which have been considered previously for naturally occurring gonadal steroids (chapter 10). Current knowledge, however, dictates that the comparison between natural and synthetic hormones must rest here:

Contraceptive preparations and their component steroids

appear to exert varied effects on carbohydrate metabolism which are often incompatible with the effects of the natural products (chapter 1). It seems preferable that individual synthetic hormones and combinations of these hormones should be considered separately, but since there is still some controversy over the nature of their effects, a deliberation of mechanistic processes may be premature. Nevertheless, several authors have already commented on this issue with particular reference to a diabetogenic influence of synthetic oestrogens.

Elevated growth hormone levels have been observed in women receiving oestrogen-progestin preparations (Spellacy & 1967b,c; 1969b; 1970b; Yen & Vela, 1968) and oestrogen alone (Spellacy et al., 1972c); and Davidson & Holzman (1973) have implicated oestrogen enhancement of growth hormone secretion as a possible mechanism of glucose intolerance. The importance of such a mechanism is uncertain since Bhatia et al. (1972) have presented evidence to suggest that progestins lower circulating growth hormone levels, and there have been reports of impaired glucose tolerance in women receiving progestin only contraceptives.

Synthetic oestrogens appear to raise the circulating level of glucocorticoids through a differential lowering of the rate of secretion and the rate of metabolic clearance. Most of the extra circulating pool is transcortin-bound and therefore relatively inactive, and it is considered that the slight increase of free glucocorticoid levels could not contribute significantly towards the impairment of glucose tolerance (Doe, Zinneman, Flink & Ulstrom, 1960; Metcalf & Beaven, 1963; Dodek, Segre

& Klaiber, 1965; Nelson, 1969).

Many other metabolic modifications are known to accompany steroid contraceptive therapy (Salhanick et al., 1969; Briggs et al., 1970) but the roles of these and other factors in diabetogenic effects must await further investigation.

Concluding remarks

In the interests of identifying and developing contraceptive preparations which do not effect glucose homeostasis, a carefully controlled, systematic clinical evaluation of the effects of synthetic oestrogens and progestins on this aspect is undoubtedly required. Until the ideal preparations become available it remains imperative that women receiving contraceptive steroids should be thoroughly screened for adverse symptoms. Several decades have yet to pass before we may become aware of the long term consequences of this kind of medication.

TABLE 5. ORALLY ADMINISTERED CONTRACEPTIVE STEROIDS

OESTROGENS

ethinyl oestradiol[•]

mestranol

quinestrol[•]

PROGESTINS

norethynodrel

norethisterone (=norethindrone)[•]

Derivatives of

norethisterone acetate[•]

19-nortestosterone

ethynodiol diacetate

lynestrenol

quingestanol acetate

norgestrel[•]

Derivatives of

medroxyprogesterone acetate[•]

17 α -hydroxyprogesterone

megestrol acetate[•]

chlormadinone acetate[•]

algestone acetophenide[•]

Derivative of testosterone

dimethisterone

[•] Also administered by pellet implant or intramuscular injection

TABLE 6. IMPLANTED AND INJECTED CONTRACEPTIVE STEROIDS

OESTROGENS

oestradiol enanthate

0 (see table 5)

PROGESTINS

progesterone

hydroxyprogesterone caproate

dydrogesterone

0 (see table 5)

TABLE 7. SCALE FOR RANKING CHANGES OF GLUCOSE TOLERANCE AND THE CIRCULATING INSULIN
RESPONSE

Ranking Symbol	Glucose Tolerance	Insulin Response	Comment
a	improvement	decrease	defined as such by the author, either with or without statistical support
b	no change	no change	no apparent or consistent changes
c	slight impairment	slight increase	the author has indicated such a trend but: (i) has not found this trend to be statistically significant, or (ii) has not statistically analysed the results
d	significant impairment	significant increase	defined as such by the author, with statistical support

TABLE 8. COMPARISON OF CONVOID AND METRULEN IN APPARENTLY NORMAL HEALTHY WOMEN OF CHILD-BEARING AGE, WITH RESPECT TO PRETREATMENT CONTROL AND THE TYPE OF GLUCOSE TOLERANCE TEST.

		C O N O V I D				G L U C O S E T O L E R A N C E				M E T R U L E N				
		Total	Pretreatment Control	Pretreatment Control	oral	iv				Total	Pretreatment Control	Pretreatment Control	oral	iv
Number of reports		16	11	4	7	11	8	5	3					
a		0	0	0	0	0	0	0	0					
b	Percentage occurrence of ranking symbols	31	36	25	43	9	0	0	0					
c		25	18	50	0	64	75	80	67					
d		44	46	25	57	27	25	20	33					

	6	6	2	4	7
Number of reports	6	6	2	4	7
Percentage occurrence of ranking symbols	a	0	0	0	0
	b	0	0	0	0
	c	50	100	25	71
	d	50	0	75	29

TABLE 9. GLUCOSE TOLERANCE DURING ARTIFICIAL MENSTRUAL CYCLES

Author	Contraceptive preparation	Duration of therapy (months)	Type of GTT	Days of Menstrual Cycle	Cycle	Changes of glucose tolerance
Pyorala et al. 1967	Serial 28 (sequential)	1	iv	0	14 21/22 27/28	Impaired days 21/22 compared with days 27/28
Taylor & Kass, 1968	Conovid (combined)	1 - 12	0	15	28	No consistent changes
Larsson-Cohn et al. 1969	Norethindrone Chlormadinone acetate (minipill)	1 - 12	iv	follicular	luteal	No consistent changes
Foss et al. 1970	Norgestrel (minipill)	> 12	0	0-5 6-7 11-16	17-28	Deterioration as cycle advances

TABLE 10. EFFECTS OF CONTRACEPTIVE PREPARATIONS ON GLUCOSE TOLERANCE
AND THE INSULIN RESPONSE^ø

Author	Duration of therapy (months)	Type of GTT	Pretreatment control	Glucose tolerance	Insulin Response
Section (1) <u>Combined preparations in apparently normal</u> <u>healthy women of child-bearing age.</u>					
Aconcene					
Bottermann et al. '67	1-3	iv	yes	c	c
Rice-Wray et al. '70	4-12	o	yes	d	-
Anovlar					
Frerichs et al. '66	12-48	iv	yes	c	-
Pehrson '70	1-12	iv	yes	b	-
Wynn & Doar '66	>3	o/iv	no	c	-
Delpregnin					
Starup '68	12	iv	yes	c	c
Conovid/E					
Danowski et al. '68	5-48	iv	yes	b	-

^ø Abbreviations used in this table are listed at the
foot of the last page of this table.

Gershberg et al. '64	3-38	o	no	d	-
Goldman et al. '69a, '70a	3	iv	yes	d	-
Javier et al. '68	>12	o	no	c	-
Javier et al. '68	1-20	o	yes	c	c
La Haba et al. '71	5-13	o	no	b	-
Peterson et al. '66	>3	o	yes	d	-
Pi-Sunyer & Oster '68	1	o	yes	c	c
Posner et al. '67a, b	2-18	iv	yes	d	-
Spellacy & Carlson '66	1	iv	yes	d	d
Spellacy et al. '67a	6	iv	yes	b	d
Spellacy et al. '68a	12	iv	yes	d	d
Spellacy et al. '69a	24-36	iv	yes	b	c
Taylor & Kass '68	1-12	o	yes	b	-
Wynn & Doar '66	>3	o/iv	no	c	-
Yen & Vela '69	3-12	o/iv	yes	d	-

Eugynon

Hillmer et al. '68	2	o/iv	yes	c	c
Neilson '72	2	iv	yes	a	c

Lyndiol

Bottermann et al. '67	1-3	iv	yes	c	b
Doorenbos et al. '69	?	o	yes	d	d
Terpstra et al. '70	3	o	yes	c	c

Waldhausl et al. '69	1	iv	yes	d	d
Wynn & Doar '69	6-24	o/iv	yes	d	d
Metrulen					
Beck & Wells '68, '69	1-2	o	yes	c	c
Javier et al. '68	1-50	o	yes	c	c
Peterson et al. '66	>3	o	yes	d	-
Pehrson '70	1-12	iv	yes	d	-
Posner et al. '67a,b	2-18	iv	yes	c	-
Spellacy et al. '71a	6	o	yes	c	c
Spellacy et al. '71b	12	o	yes	c	c
Wynn & Doar '66	>3	o/iv	no	c	-
Wynn & Doar '69	6-24	o/iv	yes	d	d
Yen & Vela '68	3	o/iv	yes	b	d
Yen & Vela '69	6	iv	yes	c	c

Norinyl

Clinch et al. '69	4	iv	yes	a	-
Clinch et al. '69	4	iv	yes	c	-
Taft et al. '69	3	o	yes	b	c
Wynn & Doar '69	6-24	o/iv	yes	d	d

Ortho-Novin

Hutcherson et al. '67	1-56	o	no	c	-
Kalkhoff et al. '69	6-17	o	yes	d	d
Spellacy et al. '70a	99	o	no	c	c

Ovral

Boshell et al. '68	1-12	o	yes	b	b
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Planovin

Vermeulen et al. '70	12-16	iv	yes	b	d
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Step-up

Wynn & Doar '69	6-24	o/iv	yes	d	d
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Volidan

Pehrson '70	1-12	iv	yes	b	-
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Unspecified

Adams et al. '71	>3	o	yes	c	d
Spellacy et al. '68b	>96	o	no	c	c
Phillips & Duffy '73	?	o	no	d	-

Section (2) Sequential preparations in apparently normal healthy women

of child-bearing age.

C-Quens

Beck & Wells '68,'69	1-2	o	yes	c	c
Javier et al. '63	1-50	o	yes	c	c
Moses & Goldzieher '70	12-24	o	yes	a	-
Puchulu et al. '67	1-12	o	yes	c	-
Spellacy et al. '68c	6	iv	yes	a	d
Spellacy et al. '70b	65-97	o	no	b	d
Spellacy et al. '71c	6	o	yes	d	d
Spellacy et al. '71d	12	o	yes	c	d
Wynn & Doar '69	6-24	o/iv	yes	d	d

Neonovum

Vermeulen et al. '70	12-16	iv	yes	a	d
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Oracon

Banks '69	3-20	?	yes	b	-
Yen & Vela '68	3	o/iv	yes	b	d

Sequens

Spellacy et al. '70a	>72	o	no	b	b
Waldhausl et al. '69	1	iv	yes	d	d

Serial 28

Pyorala et al. '67 1 iv yes ^eb -

Unspecified

Bruso et al. '70 4-16 iv yes b -
 Spellacy et al. '68b >72 o no b c
 Phillips & Duffy '73 ? o no d -

Section (3) Minipill preparations in apparently normal healthy women

of child-bearing age.

Chlormadinone acetate

Larsson-Cohn et al. '69 12 iv yes c b
 Taft et al '69 3 o yes b a
 Vermeulen et al. '70 12-16 iv yes a d

Ethynodiol diacetate

Goldman & Eckerling '72 1 iv yes b b
 Spellacy et al. '72a 6 o yes d d

Norethisterone

Larsson-Cohn et al. '69 12 iv yes c b

Norethisterone acetate

Puchulu et al. '67 3 o yes b -

Norgestrel

Foss '70 12-36 o no a -

Section (4) Depot progestin preparation in apparently normal healthy

women of child-bearing age.

Medroxyprogesterone

Goldman et al. '68 1 iv no b -
 Spellacy et al. '72b 12 o yes d d

Section (5) Combined preparations in diabetic women

of child-bearing age.

Anovlar

Lunnell & Persson '71*1 2-15 iv yes c -

Conlunett

Lunnell & Persson '71*1 2-15 iv yes c -

Conovid

Goldman & Eckerling '70a*3 2 iv yes b -

Delpregnin

Starup et al. '68*5 12 iv yes c b

Lyndiol

Doorenbos et al. '69 *2
Lunnell & Persson '71 *1

yes
yes
d
c
b
-

Metrolen

Beck & Wells '68, '69 *1

yes
d
c

Spellacy et al. '71a *3

yes
c
b

Spellacy et al. '71a *4

yes
b
a

Yen & Vela '69 *1,3

yes
c
c

Norinyl

Szabo et al. '70 *1

yes
c
-

Ortho-Novin

Lunnell & Persson '71 *1

yes
c
-

Ovral

Boshell et al. '68 *3,4

yes
b
b

Unspecified

Kalkhoff et al. '68 *3

no
c
b

Section (6) Sequential preparations in diabetic women of

child-bearing age.

C-Quens

Beck & Wells '68, '69* ¹	1-5	o	yes	d	c
Spellacy et al. '71c* ³	6	o	yes	a	d
Spellacy et al. '71d* ³	12	o	yes	a	d

Oracon

Banks '69*³

3-20	?	yes	b	-
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Section (7) Minipill preparation in diabetic women of

child-bearing age.

Ethynodiol diacetate

Goldman & Eckerling '70b* ³	3	iv	yes	b	-
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Section (8) Depot progestin preparation in diabetic women

of child-bearing age.

Medroxyprogesterone acetate

Spellacy et al. '72b* ³	12	o	yes	b	d
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Section (9) Combined preparation in women with other pathologies

of child-bearing age.

Conovid

Waine et al. '63*	1-10	?	?	c	-
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Section (10) Depot progestin preparation in women with other pathologies

of child-bearing age.

Medroxyprogesterone acetate

Blum et al. '72*	3-15	o/iv	yes	b	c
Goldman et al. '68*	1	iv	no	b	-

Section (11) Combined preparations in apparently healthy women of

post-menopausal age.

Conovid

Ajabor et al. '72	6	o	yes	d	b
Buchler & Warren '66	1	o/iv	yes	c	-
Yen & Vela '69	3-12	o/iv	yes	d	-

MetruLen

Boshell et al. '68	1-12	o	yes	b	b
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Section (12) Sequential preparations in apparently healthy women

of post-menopausal age.

Unspecified

Pyorala et al. '70	2-3	o	yes	d	b
Pyorala et al. '70	2-14	o	yes	b	a

Section (13) Combined preparation in maturity-onset diabetic women

of post-menopausal age.

Metrolen

Gold et al. '69	1	o/iv	yes	d	b
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Section (14) Minipill preparations in maturity onset diabetic women

of post-menopausal age.

Ethynodiol diacetate

Gold et al. '69	1	o/iv	yes	c	c
-----------------	---	------	-----	---	---

Unspecified

Gershberg et al. '67	?	o	?	a	b
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Section (15) Depot progestin preparation in women with other pathologies

of post-menopausal age.

Medroxyprogesterone acetate

Goldman et al. '68 ^{*s}	1	iv	no	b	-
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Ø Abbreviations:

- O: oral glucose tolerance test
- iv: intravenous glucose tolerance test
- ?: not stated
- : not measured
- Ø: measured on days 20/21; but there was no change from pretreatment control on days 15/16
- *1: gestational diabetics
- *2: latent diabetics
- *3: asymptomatic diabetics
- *4: clinical diabetics
- *5: clinical diabetics receiving insulin
- *6: rheumatoid arthritis
- *7: acromegaly
- *8: dysfunctional uterine bleeding

TABLE 11. COMPARISON OF THE EFFECTS OF DIFFERENT CONTRACEPTIVE REGIMES IN APPARENTLY NORMAL
HEALTHY WOMEN OF CHILD-BEARING AGE

		Combined	Sequential	Minipill	Depot
		<u>GLUCOSE TOLERANCE</u>			
Number of reports		55	18	8	2
Percentage occurrence of ranking symbols	a	4	17	25	0
	b	20	39	38	50
	c	42	22	25	0
	d	34	22	12	50
		<u>INSULIN RESPONSE</u>			
Number of reports		32	12	6	1
Percentage occurrence of ranking symbols	a	0	0	17	0
	b	6	8	50	0
	c	50	25	0	0
	d	44	67	33	100

TABLE 12. DIRECT COMPARISON OF COMBINED AND SEQUENTIAL
CONTRACEPTIVE PREPARATIONS IN APPARENTLY
NORMAL HEALTHY WOMEN OF CHILD-BEARING AGE

Author	Combined preparation	Sequential preparation	Conclusion
Beck & Wells, '68, '69.	Metrulen	C-Quens	No difference
Javier et al. '68	Conovid	C-Quens	No difference
Spellacy et al. '68b	?	?	Greater impair- ment with combined
Spellacy et al. '70a	Ortho-Novum	Sequens	Greater impair- ment with combined
Vermeulen et al. '70	Planovin	Neonovum	Greater impair- ment with combined
Waldhausl et al. '69	Lyndiol	Sequens	No difference
Wynn & Doar '69	various	C-Quens	No difference
Yen & Vela '68	Metrulen	Dracon	No difference

Appendix iiiTABULATION OF RESULTS

The values cited in the following tables for groups of data include:

- (i) The number of observations (n), given in parentheses,
- (ii) the mean plus or minus the standard error of the mean
- mean \pm sem.

Chapter 4. Oestrous cycle and one week ovariectomy in female rats

TABLE 13. Intraperitoneal glucose tolerance tests

	Weight (g)	Blood sugar (mg/100 ml) at times (minutes)			ipGT A		
		0	15	30			
Pro-oestrus	(10)	242.70 ± 4.25	104.80 ± 0.99	172.00 ± 2.92	132.80 ± 2.90	108.00 ± 3.31	411.00 ± 3.94
Oestrus	(12)	239.83 ± 5.59	105.66 ± 2.67	165.66 ± 3.42	134.33 ± 4.19	108.00 ± 3.55	406.66 ± 7.46
Metoestrus	(10)	239.40 ± 3.88	106.40 ± 3.63	171.20 ± 5.12	140.80 ± 4.41	122.80 ± 3.32	420.60 ± 8.79
Dioestrus	(10)	233.30 ± 4.72	106.40 ± 1.90	184.80 ± 5.35	148.80 ± 3.30	129.20 ± 2.23	451.40 ± 7.39
One week Ovariectomy	(6)	218.33 ± 2.52	104.66 ± 2.40	182.00 ± 6.90	150.33 ± 5.94	121.00 ± 1.00	445.16 ± 10.69

TABLE 14. BASAL PLASMA INSULIN LEVELS

		Weight (g)	Plasma Insulin (μ U/ml)
Pro-oestrus	(12)	252.83 ± 4.16	51.83 ± 5.54
Oestrus	(7)	242.71 ± 8.01	54.14 ± 5.42
Metoestrus	(10)	254.00 ± 6.44	43.30 ± 9.36
Dioestrus	(10)	249.90 ± 5.33	37.40 ± 4.37
One week Ovariectomy	(6)	215.83 ± 3.96	38.25 ± 5.85

Chapter 5. Age and long term ovariectomy in female rats

TABLE 15. INTRAPERITONEAL GLUCOSE TOLERANCE TESTS

	Weight (g)	Blood sugar (mg/100 ml) at times (minutes)				ipGT _A
		0	15	30	60	
Oestrus (40 weeks)	(8) 331.00 ±10.05	103.50 ± 1.76	184.50 ± 6.94	175.50 ± 9.72	134.00 ± 4.72	482.50 ±17.58
Long term Ovariectomy (40 weeks)	(7) 377.00 ± 6.48	105.14 ± 1.37	200.00 ±15.11	232.00 ±21.80	182.85 ±22.07	576.00 ±34.68

TABLE 16. BASAL PLASMA INSULIN LEVELS

		Weight (g)	Plasma Insulin (μ U/ml)
Dioestrus	(6)	311.50	35.00
(40 weeks)		± 16.79	± 3.95
Long term	(8)	353.37	44.50
Ovariectomy		± 12.53	± 3.89
(40 weeks)			

TABLE 17. INTRAVENOUS GLUCOSE TOLERANCE TESTS

		Dioestrus (40 weeks) (10)	Long term Ovariectomy (40 weeks) (4)
Weight	(g)	268.40 \pm 8.38	372.00 \pm 9.08
Blood sugar (mg/100 ml) at times(minutes)	0	102.00 \pm 2.19	109.00 \pm 4.20
	10	277.80 \pm 8.48	285.00 \pm 6.65
	20	213.80 \pm 9.87	203.50 \pm 11.89
	30	173.40 \pm 8.20	158.50 \pm 13.59
Sum of 10,20 & 30 minute values		665.00 \pm 25.63	647.00 \pm 31.39
Plasma Insulin (μ U/ml) at times(minutes)	0	24.50 \pm 2.98	31.00 \pm 4.37
	10	79.10 \pm 9.52	95.75 \pm 5.94
	20	64.50 \pm 6.64	61.00 \pm 5.67
	30	50.80 \pm 5.09	46.75 \pm 2.01
Sum of 10,20 & 30 minute values		194.40 \pm 20.69	203.50 \pm 9.56

Chapter 6. Effects of sex hormones in ovariectomized female rats

TABLE 18. INTRAPERITONEAL GLUCOSE TOLERANCE TESTS

	Weight (g)	Blood sugar (mg/100 ml) at times (minutes)			ipGT _A	
		0	15	30		60
Control (12)	275.33 ± 5.33	105.33 ± 2.66	199.33 ± 5.58	144.33 ± 3.66	113.66 ± 2.48	453.16 ± 8.83
Oestradiol (11)	259.00 ± 3.88	93.09 ± 1.62	155.63 ± 3.25	130.18 ± 4.12	98.54 ± 2.36	381.63 ± 7.99
Progesterone (10)	275.70 ± 4.59	99.20 ± 1.95	150.00 ± 3.22	125.60 ± 1.90	104.80 ± 0.99	377.60 ± 5.07
Gonadotrophins (11)	302.72 ± 6.46	100.36 ± 1.25	167.27 ± 4.90	138.54 ± 3.58	106.54 ± 1.81	409.27 ± 7.54
Testosterone (8)	298.62 ± 6.88	115.00 ± 1.96	196.00 ± 10.66	176.00 ± 9.45	149.25 ± 7.24	504.62 ± 22.63

TABLE 19. BASAL PLASMA INSULIN LEVELS

		Weight (g)	Plasma Insulin (μ U/ml)
Control	(12)	275.33 \pm 5.33	54.16 \pm 4.52
Oestradiol	(11)	258.09 \pm 4.12	80.27 \pm 7.48
Progesterone	(13)	292.07 \pm 3.26	66.46 \pm 3.31
Gonadotrophins	(21)	295.76 \pm 4.41	69.42 \pm 4.35
Testosterone	(8)	298.62 \pm 6.88	56.87 \pm 4.30

Chapter 7. Effects of sex hormones in orchidectomized male rats

TABLE 20. INTRAPERITONEAL GLUCOSE TOLERANCE TESTS

	Weight (g)	Blood sugar (mg/100 ml) at times (minutes)				ipGT _A
		0	15	30	60	
Normal (intact) (8)	245.75 ± 3.89	104.50 ± 1.40	165.50 ± 5.06	151.50 ± 4.62	123.00 ± 4.39	430.75 ± 10.64
Control (9)	271.22 ± 4.55	104.44 ± 3.22	172.44 ± 6.51	148.00 ± 6.73	110.66 ± 4.16	429.11 ± 14.25
Testosterone (9)	260.33 ± 10.03	107.11 ± 2.08	176.00 ± 4.32	156.88 ± 4.70	124.88 ± 3.75	448.88 ± 9.06
Gonadotrophins (8)	283.50 ± 7.35	105.50 ± 3.28	169.00 ± 3.98	151.00 ± 4.51	122.50 ± 5.50	434.00 ± 9.31
Progesterone (8)	292.87 ± 6.43	107.50 ± 1.18	173.00 ± 2.47	151.50 ± 1.59	127.00 ± 2.23	441.75 ± 2.21
Oestradiol (8)	251.75 ± 3.83	106.25 ± 2.60	170.00 ± 5.75	146.50 ± 6.54	118.50 ± 4.20	430.12 ± 12.86

TABLE 21. BASAL PLASMA INSULIN LEVELS

		Weight (g)	Plasma Insulin (μ U/ml)
Normal (intact)	(14)	251.64 ± 5.81	34.85 ± 2.22
Control	(8)	273.87 ± 4.09	30.50 ± 1.97
Testosterone	(8)	254.37 ± 8.27	25.50 ± 1.16
Gonadotrophins	(9)	275.22 ± 6.72	35.00 ± 3.00
Progesterone	(10)	293.30 ± 5.29	20.50 ± 2.21
Oestradiol	(9)	251.33 ± 3.40	35.00 ± 1.64

Chapter 8. Early effects of oestradiol and progesterone on
insulin secretion in female rats

TABLE 22. IMMEDIATE EFFECTS OF OESTRADIOL AND PROGESTERONE
ON INTRAVENOUS GLUCOSE TOLERANCE

		Control (9)	Oestradiol (10)	Progesterone (8)
Weight (g)		208.44 \pm 4.50	201.60 \pm 2.74	201.62 \pm 4.04
Blood sugar (mg/100 ml) at times (minutes)	0	95.33 \pm 2.60	93.60 \pm 3.52	100.00 \pm 2.90
	10	252.44 \pm 6.27	245.00 \pm 4.74	246.00 \pm 4.67
	20	207.77 \pm 7.64	209.60 \pm 4.25	201.50 \pm 4.04
	30	180.88 \pm 8.82	184.40 \pm 5.33	174.25 \pm 4.90
Total blood sugar value		736.44 \pm 22.70	732.60 \pm 15.39	721.75 \pm 12.04
Plasma insulin (μ U/ml) at times (minutes)	0	17.55 \pm 2.51	20.90 \pm 2.78	22.87 \pm 1.51
	10	86.88 \pm 11.80	94.80 \pm 7.27	99.62 \pm 9.55
	20	56.66 \pm 8.81	70.60 \pm 5.60	78.12 \pm 9.90
	30	48.22 \pm 4.88	57.40 \pm 5.10	60.00 \pm 6.52
Total plasma insulin value		209.33 \pm 18.31	243.70 \pm 15.57	260.62 \pm 24.59

TABLE 23. SIX HOUR EFFECTS OF OESTRADIOL AND PROGESTERONE
ON INTRAVENOUS GLUCOSE TOLERANCE

	Control (10)	Oestradiol (7)	Progesterone (7)
Weight (g)	216.60 \pm 3.89	216.28 \pm 4.39	216.85 \pm 3.20
Blood sugar (mg/100 ml) at times (minutes)			
0	105.00 \pm 2.29	100.28 \pm 2.90	99.71 \pm 3.89
10	273.40 \pm 5.98	251.42 \pm 4.11	252.57 \pm 3.74
20	239.00 \pm 4.91	225.42 \pm 3.37	222.85 \pm 6.30
30	220.20 \pm 4.13	202.28 \pm 3.79	197.14 \pm 6.47
Total blood sugar value	837.60 \pm 15.91	779.42 \pm 11.23	772.28 \pm 17.67
Plasma insulin (μ U/ml) at times (minutes)			
0	11.40 \pm 0.96	15.28 \pm 4.57	23.00 \pm 6.05
10	66.40 \pm 9.99	94.42 \pm 12.78	106.14 \pm 21.68
20	46.40 \pm 5.22	76.42 \pm 9.86	69.00 \pm 8.05
30	36.30 \pm 6.35	61.14 \pm 8.55	47.85 \pm 6.09
Total plasma insulin value	160.50 \pm 19.02	247.28 \pm 32.35	246.00 \pm 33.13

TABLE 24. IMMEDIATE AND SIX HOUR EFFECTS OF OESTRADIOL AND PROGESTERONE ON INSULIN
SECRETION IN VITRO

		Control	Oestradiol	Progesterone
		INSULIN SECRETION (μ U/mg dried pancreas / 30 minutes)		
IMMEDIATE	No glucose	(10) 9.69 \pm 0.73	(9) 10.14 \pm 0.87	(11) 8.57 \pm 0.52
	Glucose, 3.0 g/l	(10) 24.93 \pm 2.14	(11) 21.54 \pm 3.70	(10) 20.23 \pm 2.25
SIX HOUR	No glucose	(10) 8.62 \pm 0.71	(12) 9.87 \pm 0.58	(11) 9.22 \pm 0.79
	Glucose, 3.0 g/l	(26) 25.97 \pm 4.25	(25) 25.87 \pm 3.62	(26) 44.55 \pm 5.75

Chapter 9. Effect of melatonin on insulin secretion in male rats

TABLE 25. Effect of a constant infusion of melatonin on intravenous glucose tolerance

		Control (6)		Melatonin (7)	
Weight (g) Blood sugar (mg/100ml) at times (minutes)		298.66	± 21.11	288.00	± 15.99
	0	102.66	± 2.81	105.71	± 2.32
	10	99.66	± 4.24	101.42	± 2.91
	20	257.33	± 4.99	262.28	± 6.93
	30	215.66	± 7.16	218.85	± 8.30
	40	192.00	± 8.65	187.71	± 10.06
Total blood sugar value		879.00	± 19.21	866.00	± 20.84
Plasma insulin (μU/ml) at times (minutes)	0	23.33	± 5.07	34.28	± 4.14
	10	22.83	± 4.24	22.71	± 2.97
	20	111.00	± 12.20	98.42	± 9.39
	30	98.33	± 10.25	83.42	± 7.50
	40	66.83	± 7.27	68.57	± 8.72
Total plasma insulin value		322.33	± 19.18	307.42	± 22.97

TABLE 26. EFFECT OF MELATONIN ON INSULIN SECRETION

FROM PIECES OF PANCREAS

	CONTROL	MELATONIN
	INSULIN SECRETION (μ U/mg dried pancreas/60 minutes)	
No glucose	(7) 13.38 ± 1.54	(7) 7.76 ± 1.18
Glucose, 3.0 g/l	(9) 104.08 ± 10.82	(9) 73.40 ± 8.80

TABLE 27. EFFECT OF MELATONIN ON INSULIN RELEASE FROM AN
ISOLATED PERFUSED PANCREAS PREPARATION IN THE
PRESENCE OF GLUCOSE 100 mg/100ml

Time (minutes)	Control		Melatonin	
	(3)		(1)	
	Δ INSULIN RELEASE (μ U/ml)			
0	0	-	0	<div> <div></div> <div>Melatonin (0.1 μg/ml)</div> </div>
5	+2.6	± 3.1	-2.0	
10	-12.0	± 2.5	-5.5	
15	-0.3	± 1.2	-6.5	
20	0	± 1.0	-11.5	
25	-0.3	± 4.4	-14.5	
30	-1.3	± 0.3	-10.5	<div> <div></div> <div>Melatonin (0.5 μg/ml)</div> </div>
35	-3.6	± 1.3	-11.5	
40	-2.6	± 4.0	-9.0	
45	+0.3	± 5.7	-13.0	
50	-5.0	± 3.0	-6.0	
55	-1.6	± 6.3	-18.5	
60	-3.6	± 4.9	-15.0	
65	-7.3	± 3.8	-26.0	
70	-3.6	± 5.8	-20.5	
75	-6.6	± 6.8	-19.5	
80	-3.6	± 4.0	-18.0	
85	-3.6	± 6.0	-14.0	

Appendix i. The isolated perfused pancreas

TABLE 28. EQUILIBRATION PERIOD FOR THE PRODUCTION
OF A STEADY RELEASE OF INSULIN

	<u>No glucose</u>		<u>Glucose, 100 mg /100ml</u>		
	----- INSULIN RELEASE -----				
	(μU/ml)				
Time					
(minutes)					
5	10.0	4.6	-	-	-
10	7.2	5.4	31	22.5	25
15	7.0	5.2	34	25	17
20	4.5	7.4	33.5	29.5	22
25	4.7	8.0	35	20	19.5
30	4.9	4.9	32.5	21	28
35	3.9	6.3	31	30	25

**TABLE 29. INSULIN RELEASE IN RESPONSE TO A CONSTANT
INFUSION OF GLUCOSE, 50 mg/100 ml**

Time (minutes)	INSULIN RELEASE (μ U/ml)
0	6.3
2	4.7
3	3.3
4	4.6
8	3.7
13	2.5

**TABLE 30. INSULIN RELEASE IN RESPONSE TO A CONSTANT
INFUSION OF GLUCOSE, 100 mg/100 ml**

Time (minutes)	INSULIN RELEASE (μ U/ml)
0	14.5
1	41
2	42
3	50
4	38
9	33
14	21
19	24

TABLE 31. INSULIN RELEASE DURING LONG TERM INFUSION
OF GLUCOSE, 100 mg/100 ml

Time (minutes)	Δ INSULIN RELEASE (μ U/ml) n = 3	
	0	-
0	0	-
5	+ 2.6	\pm 3.1
10	+12.0	\pm 2.5
15	- 0.3	\pm 1.2
20	0	\pm 1.0
25	- 0.3	\pm 4.4
30	- 1.3	\pm 0.3
35	- 3.6	\pm 1.3
40	- 2.6	\pm 4.0
45	+ 0.3	\pm 5.7
50	- 5.0	\pm 3.0
55	- 1.6	\pm 6.3
60	- 3.6	\pm 4.9
65	- 7.3	\pm 3.8
70	- 3.6	\pm 5.8
75	- 6.6	\pm 6.8
80	- 3.6	\pm 4.0
85	- 3.6	\pm 6.0
90	- 2.0	\pm 3.0
95	- 4.0	\pm 5.0
100	- 6.0	\pm 3.6

TABLE 32. INSULIN RELEASE IN RESPONSE TO A CONSTANT
INFUSION OF GLUCOSE, 300 mg/100 ml

Time (minutes)	Δ INSULIN RELEASE (μ U/ml)	
	n = 3	
0	0	-
1	+14.6	\pm 2.8
2	+63.0	\pm 19.6
3	+29.3	\pm 2.6
4	+43.0	\pm 11.5
5	+33.6	\pm 19.6
6	+38.6	\pm 17.2
8	+36.3	\pm 7.0
10	+30.6	\pm 2.8
12	+40.0	\pm 4.0
14	+42.0	\pm 11.5
16	+51.6	\pm 15.9
20	+39.6	\pm 12.4
25	+59.0	\pm 25.5
30	+38.3	\pm 6.7
35	+54.6	\pm 8.1
40	+58.0	\pm 6.2
45	+58.3	\pm 14.7
50	+54.6	\pm 10.4
55	+67.3	\pm 3.2
60	+58.0	\pm 9.0

BIBLIOGRAPHY

1. ADAMS, F.W., MUNDAY, M.J., OAKLEY, N.W. & WYNN, V. (1971). The effect of oral contraceptives and glucocorticoids on insulin response to intensive islet-cell stimulation. Diabetologia 7: 477-478.
2. AJABOR, L.N., TSAI, C.C., VELA, P. & YEN, S.S.C. (1972). Effect of exogenous estrogen on carbohydrate metabolism in postmenopausal women. Am. J. Obstet. Gynec. 113: 383-387.
3. ALBANO, J. & EKINS, R.P. (1970). The attainment of high sensitivity and precision in radioimmunoassay techniques as exemplified in a simple assay of serum insulin. In In vitro procedures with radioisotopes in medicine, pp. 491-512. Vienna: International Atomic Energy Agency.
4. ALBANO, J.D.M., EKINS, R.P., MARITZ, G. & TURNER, R.C. (1972). A sensitive precise radioimmunoassay of serum insulin relying on charcoal separation of bound and free hormone moieties. Acta endocr., Copnh. 70: 487-509.
5. ALBYASSINE, H. & GARDINER, R.J. (1972). A simple method of visualizing the islets of Langerhans within the pancreas and during their isolation. Endocrinology 91: 1350-1354.
6. ANDERSON, E. & LONG, J. (1947). The effect of hyperglycaemia on insulin secretion as determined with the isolated rat pancreas in a perfusion apparatus. Endocrinology 40: 92-97.
7. ANDERSSON, A. & HELLERSTRÖM, C. (1972). Metabolic characteristics of isolated pancreatic islets in tissue culture. Diabetes 21: (suppl.2) 546-554.

8. ANGERWALL, L. (1959). Alloxan diabetes and pregnancy in the rat; effects on offspring. Acta endocr., Copnh. 31: (suppl.44), 1-86.
9. ANTONINI, F.M. & PETRUZZI, E. (1970). Sexual disturbances in male diabetics. In Abstracts of the 7th Annual Meeting of the European Association for the Study of Diabetes. Novo Service. Abstract 218.
10. ARQUILLA, E.R., RODARI, T. & GOSIGNANI, G. (1960). Immunological determination of plasmatic insulin: methodological aspects. Archs Sci. med. 109: 471-479.
11. ARQUILLA, E.R. & STAVITSKY, A.B. (1956). The production and identification of antibodies to insulin and their use in assaying insulin. J. clin. Invest. 35: 458-466.
12. ASHFORD, W.R., CAMPBELL, J., DAVIDSON, J.K., FISHER, A.M., HAIST, R.E., LACEY, A.H., LIN, B., MARTIN, J.M., MORLEY, N.H., RASTOGI, K.S. & STORUIK, W.O. (1969). A consideration of methods of insulin assay. Diabetes 18: 828-833.
13. ASINELLI, C. & CASASSA, P. (1937). Studi sul metabolismo degli idrati di carbonio durante il ciclo mestruale. I. Ricerche in donne normali. Archivo per le Scienze Mediche 64: 431-449.
14. ATKINS, G.L. (1971). Investigation of some theoretical models relating the concentrations of glucose and insulin in plasma. J. theor. Biol. 32: 471-494.
15. ATKINS, T.W. (1972). Further studies on the isolated islets of Langerhans of obese hyperglycaemic mice and their normal lean littermates. Ph.D. thesis. University of Aston in Birmingham.

16. ATKINS, T. & THORNBURN, C.C. (1971). The effect of some anaesthetics on the blood-sugar of mice. Comp. gen. Pharmac. 2: 36-42.
17. AYNLEY-GREEN, A. & ALBERTI, K.G.M.M. (1972). Serum-insulin or plasma-insulin? Lancet i: 318-319.
18. BAILEY, C.J., ATKINS, T.W. & MATTY, A.J. (1973). Melatonin inhibition of insulin secretion in the rat and mouse. Horm. Res. In press.
19. BANKS, A.L. (1969). Do oral contraceptives affect diabetes? J. Reprod. Med. 3: 169-170.
20. BASABE, J.C., CHERI, R.A. & FOGLIA, V.G. (1969). Action of sex hormones on insulinemia of castrated female rats. Proc. Soc. exp. Biol. Med. 130: 1159-1161.
21. BASABE, J.C., LOPEZ, N.L., VIKTORA, J.K. & WOLFF, F.W. (1971a). Insulin secretion studied in the perfused rat pancreas. I. Effect of tolbutamide, leucine and arginine; their interaction with diazoxide, and relation to glucose. Diabetes 20: 449-456.
22. BASABE, J.C., LOPEZ, N.L., VIKTORA, J.K. & WOLFF, F.W. (1971b). Insulin secretion studied in the perfused rat pancreas. II. Effect of glucose, glucagon, 3'5' adenosine monophosphate, theophylline, imidazole and phenoxybenzamine; their interaction with diazoxide. Diabetes 20: 457-466.
23. BATES, R.W. & GARRISON, M.M. (1971). Solid-phase radioimmunoassay of insulin. In Laboratory diagnosis of endocrine diseases, pp. 332-334. Eds. F.W. Sunderman & F.W. Sunderman, jnr. London: Adam Hilger.
24. BEACH, E.F., BRADSHAW, P.J. & BLATHERWICK, N.R. (1951). Alloxan diabetes in the albino rat as influenced by sex. Am. J. Physiol. 166: 364-373.

25. BEACH, E.F., CULLIMORE, O.S. & BRADSHAW, P.J. (1957) Pancreatectomy diabetes in the rat as influenced by strain, age and sex. Am. J. Physiol. 191: 19-22.
26. BEATON, J.H. (1964). Menstrual regulation of sequential hormone therapy. West. J. Surg. 72: 160-163.
27. BECK, L.V., ZAHARKO, D.S., ROBERTS, N., McNEIL, T., KING, C. & BLANKENBAKER, B. (1964). Insulin assay by combined use of I-131-labelled insulin, anti-insulin serum and insulinase. Life Sci. 3: 545-550.
28. BECK, P. (1969a). Effects of gonadal hormones and contraceptive steroids on glucose and insulin metabolism. In Metabolic effects of gonadal hormones and contraceptive steroids, pp. 97-125. Eds. H. A. Sahanick, D.M. Kipnis & R.L. Vande Wiele. New York & London: Plenum Press.
29. BECK, P. (1969b). Progestin enhancement of the plasma insulin response to glucose in rhesus monkeys. Diabetes 18: 146-152.
30. BECK, P. (1971). Estriol modification of the effects of progesterone and human chorionic somatomammotropin on glucose tolerance and plasma insulin in rhesus monkeys. Diabetes 20: 271-275.
31. BECK, P. (1973). Contraceptive steroids: modifications of carbohydrate and lipid metabolism. Metabolism 22: 841-855.
32. BECK, P. & WELLS, S. (1968). Plasma insulin responses to glucose in normal and subclinical diabetic women treated with oral contraceptive agents. Diabetes 17: (suppl.1), 307-308.

33. BECK, P. & WELLS, S.A. (1969). Comparison of the mechanisms underlying carbohydrate intolerance in subclinical diabetic women during pregnancy and during post-partum oral contraceptive steroid treatment. J. clin. Endocr. Metab. 29: 807-818.
34. BENJAMIN, F. & CASPER, D.J. (1966). Alterations in carbohydrate metabolism induced by progesterone in cases of endometrial carcinoma and hyperplasia. Am. J. Obstet. Gynec. 94: 991-996.
35. BENJAMIN, F. & CASPER, D.J. (1966). Oral versus intravenous glucose tolerance tests during pregnancy. Am. J. Obstet. Gynec. 94: 566-570.
36. BERDANIER, C.D., MARSHALL, M.V. & MOSER, P. (1971). Age changes in the level of serum immunoreactive insulin in three strains of rats. Life Sciences 10: 105-109.
37. BERGQVIST, N. (1954). The influence of pregnancy on diabetes. Acta Endocr., Copnh. 15: 166-181.
38. BERIC, V.B. & KARANFILSKI, B. (1957). Die Wirkung des Östradiols auf den Blutzuckerspiegel und auf die Kurve der alimentär hervorgerufenen Hyperglykämie. Gynaecologia 143: 400-409.
39. BERSON, S.A. & YALOW, R.S. (1959). Quantitative aspects of the reaction between insulin and insulin-binding antibody. J. clin. Invest. 38: 1996-2016.
40. BERSON, S.A. & YALOW, R.S. (1959). Species-specificity of human anti-beef, pork insulin serum. J. clin. Invest. 38: 2017-2025.
41. BERSON, S.A. & YALOW, R.S. (1963). Antigens in insulin: determinants of specificity of porcine insulin in man. Science, N.Y. 139: 844-845.

42. BERSON, S.A. & YALOW, R.S. (1964). Immunoassay of protein hormones. In The hormones, vol. IV, pp. 557-630. Eds. G. Pincus, K.V. Thimann & E.B. Astwood. New York and London: Academic Press.
43. BERSON, S.A. & YALOW, R.S. (1968). Principles of immunoassay of peptide hormones in plasma. In Clinical endocrinology, vol. II. pp. 699-720. Eds. E.B. Astwood & C.E. Cassidy. New York and London: Grune and Stratton.
44. BERSON, S.A., YALOW, R.S., GLICK, S.M. & ROTH, J. (1964). Immunoassay of protein and peptide hormones. Metabolism 13: 1135-1153.
45. BERTOLINI, A.M. (1969). Gerontological metabolism. Springfield, Illinois: Charles C. Thomas.
46. BESCH, P.K., VORYS, N., ULLERY, J.C., STEVENS, J. & BARRY, R. (1965). Some systematic effects of a progestational agent, (ethynodiol diacetate and mestranol). Metabolism 14: 387-397.
47. BESSMAN, S.P. & SCHULTZ, R.D. (1972). Sugar electrode sensor for the "artificial pancreas". Horm. Metab. Res. 4: 413-417.
48. BHATIA, S.K., MOORE, D. & KALKHOFF, R.K. (1972). Progesterone suppression of the plasma growth hormone response. J. clin. Endocr. Metab. 35: 364-369.
49. BILLEWICZ, W.Z., ANDERSON, J. & LIND, T. (1973). New index for evaluation of oral glucose tolerance test results. Br. med. J. 1: 573-577.
50. BIRKINSHAW, V.J., RANDALL, S.S. & RISDALL, P.C. (1962). Formation of precipitin lines between insulin and anti-insulin serum produced in sheep and in guinea pigs. Nature, Lond. 193: 1089-1090.

51. BIRNBAUMER, L. & RODBELL, M. (1969). Adenyl cyclase in fat cells. II. Hormone receptors. J. biol. Chem. 244: 3477-3482.
52. BISHOP, O.N. (1966). Statistics for biology: a practical guide for the experimental biologist. London: Longman.
53. BLEICHER, S.J., O'SULLIVAN, J.B. & FREINKEL, N. (1964). Carbohydrate metabolism in pregnancy. V. The interrelations of glucose, insulin and free fatty acids in late pregnancy and postpartum. New Engl. J. Med. 271: 866-872.
54. BLUM, I., KARP, M. & LARON, Z. (1972). Influence of medroxyprogesterone acetate therapy on carbohydrate metabolism in acromegaly. Israel J. med. Sc. 8: 881-882.
55. BORENSTEIN, R., STAHL, N.L., USAF, L. & GREENBLATT, R.B. (1972). Effects of sex steroids on lipid levels. J. Am. Ger. Soc. 20: 369-376.
56. BOSHELL, B.R., RODDAM, R.F., McADAMS, G.L. & FOX, O.J. (1968). The effects of oral contraceptives on glucose tolerance. J. Reprod. Fert. suppl. 5: 77-88.
57. BOTTERMAN, P., DIETERLE, P., HOCHHEUSER, W., HORN, K., KOPETZ, K., SCHLEYPEN, K. & SCRIBA, P.C. (1967). Zur Frage der endokrinen Nebenwirkung von Ovulationshemmern. München Med. Wschr. 13: 685-691.
58. BOTTIGLIONI, F. & FLAMIGNI, C. (1963). Influence de differents traitements hormonaux sur l'ilot de Langerhans. Gynec. Obstet. 62: 607-630.
59. BOUMAN, P.R. (1960). Release of insulin from isolated rat pancreas in vitro. Acta endocr., Copnh. 35: 560-567.

60. BOYNS, D.R., CROSSLEY, J.N., ABRAMS, M.E., JARRETT, R.J. & KEEN, H. (1960). Oral glucose tolerance and related factors in a normal population sample. I. Blood sugar, plasma insulin, glyceride, and cholesterol measurements and the effects of age and sex. Br. med. J. 1: 595-598.
61. BRIGGS, M.H., PITCHFORD, A.G., STANIFORD, M., BARKER, H.M. & TAYLOR, D. (1970). Metabolic effects of steroid contraceptives. Adv. steroid biochem. pharmacol. 2: 111-222.
62. BRODAL, B.P. (1971a). The influence of haemolysis on the radioimmunoassay of insulin. Scand. J. clin. Lab. Invest. 28: 287-290.
63. BRODAL, B.P. (1971b). Evidence of an enzymatic degradation of insulin in blood in vitro. Eur. J. Biochem. 18: 201-206.
64. BRUSO, O.J., DeLELLIS, R.H., RIDRUEGIO, O. & MENDIZABAL, B. (1970). Modifications produced in glucose tolerance by prolonged administration of oral gestagens. Int. Congr. Series no. 209, p.28. Amsterdam: Excerpta Medica Foundation.
65. BUCHANAN, K.D. & MCCARROLL, A.M. (1971). Comparison of methods of separation of free from bound hormone in the radioimmunoassay of insulin and glucagon. In Radioimmunoassay methods, pp. 266-272. Eds. K.E. Kirkham & W.H. Hunter. Edinburgh and London: Churchill Livingstone.
66. BUCHLER, D. & WARREN, J. (1966). Effects of estrogen on glucose tolerance. Am. J. Obstet. Gynec. 95: 479-483.

67. BURR, I.M., STAUFFACHER, W., BALANT, L., RENOLD, A.E. & GRODSKY, G.M. (1969). Regulation of insulin release in perfused pancreatic tissue. Acta diabet. lat. 6: (suppl.1), 580-596.
68. BURT, R.L. (1956). Peripheral utilization of glucose in pregnancy. III. Insulin tolerance. Obstet. Gynec. 7: 658-664.
69. BURT, R.L., LEAKE, N.H. & RHYNE, A.L. (1969). Glucose tolerance during pregnancy and the puerperium. Obstet. Gynec. 33: 634-641.
70. BUTTERFIELD, W.J.H. (1964). Summary of results of the Bedford Diabetic survey. Proc. R. Soc. Med. 57: 196-200.
71. CALLOWAY, N.O. & KUJAK, R. (1971). Age and the kinetics of response to sugars and insulin. J. Am. ger. Soc. 19: 122-130.
72. CAMPBELL, N., PYKE, D.R. & TAYLOR, K.W. (1971). Oral glucose tolerance tests in pregnant women with potential diabetes, latent diabetes and glycosuria. J. Obstet. Gynaec. Br. Commonw. 76: 498-504.
73. CANTRELL, J.W., HOCHHOLZER, J.M. & FRINGS, C.S. (1972). Effect of hemolysis on the apparent concentration of insulin in plasma. Clin. Chem. 18: 1423-1425.
74. CARDEZA, A.F. & RODRIGUEZ, R.R. (1949). Accion local del estradiol sobre los islotes de Langerhans. Rev. Soc. argent. Biol. 25: 178-182.
75. CATT, K.J. (1971). An ABC of endocrinology. London: The Lancet Ltd.

76. CATT, K.J., NIALL, H.D. & TREGGEAR, G.W. (1967). A solid phase disk radioimmunoassay for human growth hormone. J. Lab. clin. Med. 70: 820-830.
77. CATT, K.J. & TREGGEAR, G.W. (1967). Solid-phase radioimmunoassay in antibody-coated tubes. Science, N.Y. 158: 1570-1572.
78. CATT, K.J., TREGGEAR, G.W. & BURGER, H.G. (1970). Radioimmunoassay of polypeptide hormones in antibody-coated tubes. In In vitro procedures with radioisotopes in medicine, pp.633-646. Vienna: International Atomic Energy Agency.
79. CEGRELL, L. (1968). The occurrence of biogenic monoamines in the mammalian endocrine pancreas. Acta physiol. scand. suppl. 314: 1-46.
80. CERASI, E. & LUFT, R. (editors) (1970). Pathogenesis of diabetes mellitus. New York: John Wiley & Sons, Inc.
81. CHANDALIA, H.B. & BOSHELL, B.R. (1970). Diagnosis of diabetes: the size and nature of carbohydrate load. Diabetes 19: 863-869.
82. CHAMBERLAIN, M.J. & STIMMLER, L. (1967). The renal handling of insulin. J. clin. Invest. 46: 911-919.
83. CHARLES, M.A., FANSKA, R., SCHMID, F.G., FORSHAM, P.H. & GRODSKY, G.M. (1973). Adenosine 3'5'-monophosphate in pancreatic islets: glucose-induced insulin release. Science, N.Y. 179: 569-571.
84. CHICK, W.L., LAURIS, V., FLEWELLING, J.H., ANDREWS, K.A. & WOODRUFF, J.M. (1973). Effects of glucose on beta cells in pancreatic monolayer cultures. Endocrinology 92: 212-218.

85. CHIERI, R.A., PIVETTA, O.H. & FOGLIA, V.G. (1969).
Altered ovulation pattern in experimental diabetes.
Fert. Steril. 20: 661-666.
86. CHLOUVERAKIS, C., JARRETT, R.J. & KEEN, H. (1967).
Glucose tolerance, age, and circulating insulin.
Lancet 1: 806-809.
87. CIACCIO, L.A. & LISK, R.D. (1972). Effect of hormone
priming on retention of ^3H -oestradiol by males and
females. Nature New Biology 236: 82-83.
88. CLARK, J.L. & STEINER, D.F. (1969). Insulin biosynthesis
in the rat: demonstration of two proinsulins. Proc.
nath. Acad. Sci. U.S.A. 62: 278-285.
89. CLINCH, J., TURNBULL, A.C. & KHOSLA, T. (1969). Effect
of oral contraceptives on glucose tolerance. Lancet 1:
857-858.
90. COHEN, S.L. (1971). The excretion of "labile" oestrogens
during human pregnancy. II. Diabetic pregnancy. Acta
endocr., Copnh. 67: 687-697.
91. COHN, R.E. (1971). Oral contraceptive agents and the
glucose tolerance problem. Pa Med. 74: 62-64.
92. COLE, H.S. (1973). Carbohydrate metabolism in children:
(1) Blood sugar, serum insulin, and growth hormone
during oral glucose tolerance tests; (2) Blood sugar
during the cortisone glucose tolerance test. Metabolism
22: 289-293.
93. COLT, E.W.D., BECKER, K.L. & QUATRALE, A.C.W. (1971).
A rapid radioimmunoassay for insulin. Am. J. clin.
Path. 55: 40-42.

94. COOPER, K.J. & HAYNES, N.B. (1969). Modification of the oestrous cycle of the underfed rat associated with the time of feeding. J. Reprod. Fert. 19: 577-579.
95. COORE, H.G. & RANDLE, P.J. (1964). Regulation of insulin secretion studied with pieces of rabbit pancreas incubated in vitro. Biochem. J. 93: 66-78.
96. CORTE, M.D., ROMANO, S., VOEGHELIN, M.R. & SERIO M. (1970). On a mathematical model for the analysis of the glucose tolerance curve. Diabetes 19: 445-449.
97. COSTRINI, N.V. & KALKHOFF, R.K. (1971). Relative effects of pregnancy, estradiol, and progesterone on plasma insulin and pancreatic islet insulin secretion. J. clin. Invest. 50: 992-999.
98. COTES, P.M. (1969). Collaborative study of estimates by radioimmunoassay of insulin concentrations in plasma samples examined in groups of 5 or 6 laboratories. J. Endocr. 45: 137-138.
99. CRAMER, H.I. (1942). The influence of menstruation on carbohydrate tolerance in diabetes mellitus. Can. med. Ass. J. 47: 51-55.
100. CROCKFORD, P.M., HARBECK, R.J. & WILLIAMS, R.H. (1966). Influence of age on intravenous glucose tolerance and serum immunoreactive insulin. Lancet 1: 465-467.
101. CSABA, G. & BARATH, P. (1971). Are Langerhans' islets influenced by the pineal body? Experientia 27: 962.
102. CURRY, D.L., BENNETT, L.L. & GRODSKY, G.M. (1968). Dynamics of insulin secretion by the perfused rat pancreas. Endocrinology 83: 572-584.
103. CUSHMAN, P. (1969). Influence of gonadotropins on adrenal cortical secretion. Clin. Res. 17: 282.

104. CZERNIAK, P., CHLEBOWSKI, J., KULCAR, S. & BORUCHOWSKI, S. (1970). Insulin radioimmunoassay for clinical research in psychiatric, pancreatic, cirrhotic and irradiated patients. In In vitro procedures with radioisotopes in medicine, pp. 549-565. Vienna: International Atomic Energy Agency.
105. DAANE, T.A. & PARLOW, A.F. (1971). Periovulatory patterns of rat serum follicle stimulating hormone and luteinizing hormone during the normal estrous cycle: effects of pentobarbitol. Endocrinology **88**: 653-663.
106. D'AMOUR, F.E., BLOOD, F.R. & BELDEN, D.A. (1969). Manual for laboratory work in mammalian physiology, 3rd edn. Chicago and London: The University of Chicago Press.
107. DANOWSKI, T.S., SABEH, G., ALLEY, R.A., ROBBINS, T.J., TSAI, C.T. & SEKARAN, K. (1968). Glucose tolerance prior to and during therapy with contraceptive steroids. Clin. Pharmac. Ther. **9**: 223-227.
108. DAVIDSON, M.B. & HOLZMAN, G.B. (1973). Role of growth hormone in the alteration of carbohydrate metabolism induced by oral contraceptive agents. J. clin. Endocr. Metab. **36**: 246-255.
109. DAVIS, M.E., FUGO, M. & LAWRENCE, K.C. (1947). Effects of alloxan diabetes on reproduction in the rat. Proc. Soc. exp. Biol. Med. **66**: 638-642.
110. DEAN, F.D., COLE, P.M. & CHESTER JONES, I. (1959). Relative rates of corticosterone secretion in intact and gonadectomized male and female rats. J. Endocr. **18**: iii-iv.

111. DEANESLY, R. (1966). The endocrinology of pregnancy and foetal life. In Marshall's physiology of reproduction, 3rd edn, vol.3, pp.891-1063. Ed. A.S. Parkes. London: Longmans, Green and Company Ltd.
112. DEBELJUK, L., VILCHEZ, J.A., SCHNITMAN, M.A., PAULUCCI, O.A. & FEDER, V.M. (1971). Further evidence for a peripheral action of melatonin. Endocrinology 89: 1117-1119.
113. DEPARTMENT OF EMPLOYMENT AND PRODUCTIVITY (1968). Code of practice for the protection of persons exposed to ionising radiations in research and teaching, 2nd edn. London: Her Majesty's Stationery Office.
114. DEUEL, H.J., GULICK, M., GRUNEWALD, C.F. & CUTLER, C.H. (1934). Sexual variations in carbohydrate metabolism. J. biol. Chem. 104: 519-530.
115. DICKENS, F., RANDLE, P.J. & WHELAN, W.J. (editors) (1968). Carbohydrate metabolism and its disorders, vols 1 & 2. London and New York: Academic Press.
116. DICZFALUSY, E. (1968). Mode of action of contraceptive drugs. Am. J. Obstet. Gynec. 100: 136-163.
117. DODEK, O.I., SEGRE, E.J. & KLAIBER, E.L. (1965), Effects of Enovid on cortisol metabolism. Am. J. Obstet. Gynec. 93: 173-178.
118. DOE, R.P., ZINNEMAN, H.H., FLINK, E.B. & ULSTROM, R.A. (1960). Significance of the concentration of nonprotein-bound plasma cortisol in normal subjects, Cushing's syndrome, pregnancy, and during estrogen therapy. J. clin. Endocr. Metab. 20: 1484-1492.

119. DONOVAN, B.T. (1970). Mammalian neuroendocrinology. London: McGraw-Hill Book Co. Ltd.
120. DOORENBOS, H., REITSMA, W.D., TERPSTRA, P. & VISSER, J.W.E. (1969). Effect of an oral contraceptive on glucose tolerance and the levels of immunoreactive insulin, free fatty acids and triglycerides. Acta endocr., Copnh. 61: (suppl.138), 157.
121. DOVE, G.A., MORLEY, F., BATCHELOR, A. & LUNN, S.F. (1971). Oestrogenic function in postmenopausal women. J. Reprod. Fert. 24: 1-8.
122. DRILL, V.A. (1966). Oral contraceptives. New York: McGraw-Hill Book Company.
123. DRURY, M.I., SWEENEY, E.C. & UACONAILL, D. (1972). Blood glucose determination by dextrostix/reflectance meter system. Ir. J. med. Sci. 141: 181-186.
124. DRURY, R.A.B. & WALLINGTON, E.A. (1967). Carleton's histological technique, 4th edn. New York and Toronto: Oxford University Press.
125. DUFFY, T., PHILLIPS, N. & PELLEGRIN, F. (1973). Review of glucose tolerance - a problem in methodology. Am. J. Med. Sci. 265: 117-133.
126. ECKSTEIN, P. (1962). Ovarian physiology in the non-pregnant female. In The ovary, vol. 1, pp. 311-359. Ed. Sir S. Zuckerman. New York & London: Academic Press.
127. ECKSTEIN, P. & ZUCKERMAN, Sir S. (1965a). Changes in the accessory reproductive organs of the non-pregnant female. In Marshall's physiology of reproduction, 3rd edn, vol.1, part 1, pp. 543-654. Ed. A.S. Parkes. London: Longmans.

128. ECKSTEIN, P. & ZUCKERMAN, Sir S. (1965b). The oestrous cycle in the mammalia. In Marshall's physiology of reproduction, 3rd edn, vol.1, part 1, pp. 226-396. Ed. A.S. Parkes. London: Longmans.
129. EHRLICH, J.C. & RATNER, I.N. (1961). Amyloidosis of the islets of Langerhans. A restudy of islet hyalin in diabetic and nondiabetic individuals. Am. J. Path. 38: 49-59.
130. EKINS, R.P., NEWMAN, G.B. & O'RIORDAN, J.L.H. (1968). Theoretical aspects of "saturation" and radioimmunoassay. In Radioisotopes in medicine: in vitro studies, pp. 59-100. Eds R.L. Hayes, F.A. Goswitz & B.E.P. Murphy. AEC Symp. Series no.13. Oak Ridge, Tennessee: Atomic Energy Commission.
131. ELLIS, L.C. (1972). Inhibition of rat testicular androgen synthesis in vitro by melatonin and serotonin. Endocrinology 90: 17-28.
132. EXTON, J.H. (1972). Gluconeogenesis. Metabolism 21: 945-990.
133. FAERMAN, I., VILAR, O., RIVAROLA, M.A., ROSNER, J.M., JADZINSKY, M.N., FOX, D., PEREZ LLORET, A., BERNSTEIN-HAHN, L. & SARACENI, D. (1972). Impotence and diabetes. Studies of androgenic function in diabetic impotent males. Diabetes 21: 23-30.
134. FAJANS, S.S., FLOYD, J.C., CONN, J.W. & PEK, S. (1970). The course of asymptomatic diabetes of children adolescents and young adults. In Early diabetes, pp. 377-384. Eds R.A. Camerini-Davalos & H.S. Cole. New York: Academic Press.
135. FEDER, H.H., RESKO, J.A. & GOY, R.W. (1968). Progesterone levels in arterial plasma of pre-ovulatory and ovariectomized rats. J. Endocr. 41: 563-569.

136. FELDMAN, J.M., BOYD, A.E. & LEOVITZ, H.E. (1971). Structural determinants of catecholamine action on in vitro insulin release. J. Pharmac. exp. Ther. 176: 611-621.
137. FELDMAN, J.M. & LEOVITZ, H.E. (1970). Serotonin inhibition of in vitro insulin release from golden hamster pancreas. Endocrinology 86: 66-70.
138. FELDMAN, J.M. & LEOVITZ, H.E. (1971). The nature of the interaction of amines with the pancreatic beta cell to influence insulin secretion. J. Pharmac. exp. Ther. 179: 56-65.
139. FELDMAN, J.M. & LEOVITZ, H.E. (1972). Structural determinants of indole amine action on in vitro insulin release. Endocrinology 91: 809-816.
140. FELDMAN, J.M. & LEOVITZ, H.E. (1973). Role of pancreatic monoamines in the impaired insulin secretion of the fasting state. Endocrinology 92: 1469-1474.
141. FELDMAN, J.M. & QUICKEL, K.E. (1973). Effects of amino acids, epinephrine and heparin upon the radioimmunoassay of insulin in plasma. Diabetes 22: 9-15.
142. FELDMAN, J.M., QUICKEL, K.E. & LEOVITZ, H.E. (1972). Potentiation of insulin secretion in vitro by serotonin antagonists. Diabetes 21: 779-788.
143. FENICHEL, R.L., PURSE, R.M., ALBURN, H.E. & EDGREN, R.A. (1969). Effects of oral contraceptives and their components on glucose tolerance in rats. Acta endocr. Copnh. 62: 438-448.
144. FERREBEE, J.W., JOHNSON, B.B., MITHOEFER, J.C. & GARDELLA, J.W. (1951). Insulin and adrenocorticotropin labelled with radio-iodine. Endocrinology 48: 277-283.

145. FERRIMAN, D.G. & GILLILAND, I.C. (1968). A synopsis of endocrinology and metabolism. Bristol: John Wright & Sons, Ltd.
146. FIORETTI, P., GENAZZANI, A.R., AUBERT, M.L., GRAGNOLI, G. & PUPILLO, A. (1970). Correlations between human chorionic somato-mammotrophin (lactogen), immunoreactive insulin, glucose and lipid fractions in plasma of pregnant women. J. Obstet. Gynaec. Br. Commonw. 77: 745-751.
147. FISHER, R.B. (1955). The absorption of water and of some small solute molecules from the isolated small intestine of the rat. J. Physiol. 130: 655-664.
148. FITZGERALD, M.G., MALINS, J.M., O'SULLIVAN, D.J. & WALL, M. (1961). The effect of sex and parity on the incidence of diabetes mellitus. Q. Jl. Med. 30: 57-70.
149. FOGLIA, V.G., CHERI, R.A. & RAMOS, M. (1970). Mechanisms of disturbances during pregnancy in the diabetic female rat. Horm. Metab. Res. 2: 76-80.
150. FOGLIA, V.G., PENHOS, J.C. & CARDEZA, A.F. (1954). Accion de las hormonas sexuales sobre los islotes y la diabetes de ratas hipofisoprivas con pancreatectomia parcial. Rev. Soc. argent. Biol. 30: 100-107.
151. FOGLIA, V.G., ROSNER, J.M., RAMOS, M.C. & LEMA, B.E. (1969). Sexual disturbances in the male diabetic rat. Horm. Metab. Res. 1: 72-77.
152. FOGLIA, V.G., SCHUSTER, N. & RODRIGUEZ, R.R. (1947). Sex and diabetes. Endocrinology 41: 428-434.
153. FOSS, G.L., HOLTON, J.B. & LEWIS, F.J.W. (1970). Effects of long-term microdosage of Norgestrel on glucose tolerance and serum transaminase levels. J. Reprod. Fert. 23: 185-188.

154. FRASCHINI, F., COLLU, R. & MARTINI, L. (1971). Mechanisms of inhibitory actions of pineal principles on gonadotropin secretion. In The pineal gland, pp. 259-278. London: Churchill Livingstone.
155. FRASER, T.R. (1969). Discussion. Non-hormonal non-specificity: interference with primary hormone-antibody reaction by salts and other solutes. In Protein and polypeptide hormones, pp.554-556. Ed. M. Margoulies. Int. Congr. Series no.161. Amsterdam: Excerpta Medica Foundation.
156. FREEDLENDER, A.E. (1969). Practical and theoretical advantages of the use of I^{125} in radioimmunoassay. In Protein and polypeptide hormones, pp. 351-353. Ed. M. Margoulies. Int. Congr. Series no.161. Amsterdam: Excerpta Medica Foundation.
157. FRERICHS, H., CREUTZFELDT, C. & CREUTZFELDT, W. (1968). Inhibitors of insulin secretion. Acta diabet. lat. 5: (suppl.1), 105-123.
158. FRERICHS, H., GROTE, S., SEVERIDT, E. & CREUTZFELDT, W. (1966). Änderungen der Glucoseassimilation in Verlauf des Menstruationcyclis und während der Behandlung mit einem Ovulationshemmer (Äthinylnortestosteronacetat. Anovlar). Symp. Otsch. Ges. Endokrinologie 12: 123-126.
159. FROLKIS, V.V., BOGATSKAYA, L.N., BOGUSH, S.U. & SHEVCHUK, V.G. (1971). Content and activity of insulin in the blood and the sensitivity of tissues to it during aging. Geriatrics 26: 118-129.
160. FUCHS, F. & KLOPPER, A. (1971). Endocrinology of pregnancy. New York, Evanston, and London: Harper & Row, Publishers.

161. FURMAN, R.H. (1969). Gonadal steroid effects on serum lipids. In Metabolic effects of gonadal hormones and contraceptive steroids, pp. 246-264. Eds. H.A. Salhanick, D.M. Kipnis & R.L. Vande Wiele. New York and London: Plenum Press.
162. FUSSGÄNGER, R.D., GOBERNA, R., VOIGT, K.H., LAUBE, H. & PFEIFFER, E.F. (1971). Sex specific differences in the metabolic effects of the progestin chlormadinone acetate in subtotally pancreatectomized rats. Acta endocr., Copnh. 67: (suppl.155), 183.
163. GAGLIARDINO, J.J. & HERNANDEZ, R.E. (1971). Circadian variation of the serum glucose and immunoreactive insulin levels. Endocrinology 88: 1529-1531.
164. GALA, R.R. & WESTPAL, U. (1965). Corticosteroid-binding globulin in the rat: studies on the sex difference. Endocrinology 77: 841-851.
165. GARUFI, G. & RUGGERI, G. (1933). Curva glicemica e ciclo mestruale. Monitore Obstetrico-Ginecologico di Endocrinologia e Metabolismo 5: 557-596.
166. GAY, V.L. & MIDGLEY, A.R. (1969). Response of the adult rat to orchidectomy and ovariectomy as determined by LH radioimmunoassay. Endocrinology 84: 1359-1364.
167. GELEHRTER, T. (1973). Mechanisms of hormonal induction of enzymes. Metabolism 22: 85-100.
168. GENUTH, S., FROHMAN, L.A. & LEBOVITZ, H.E. (1965). A radioimmunological assay method for insulin using insulin-I¹²⁵ and gel filtration. J. clin. Endocr. Metab. 25: 1043-1049.
169. GERSHBERG, H., HULSE, M. & JAVIER, Z. (1967) Improvement of glucose tolerance with estrogen treatment in maturity-onset diabetes. Int. Congr. Series no.140, p.129. Amsterdam: Excerpta Medica Foundation.

170. GERSHBERG, H., JAVIER, Z. & HULSE, M. (1964). Glucose tolerance in women receiving an ovulatory suppressant. Diabetes 13: 378-382.
171. GERSHBERG, H., JAVIER, Z., HULSE, M. & COHANE, J. (1967). Improvement of glucose tolerance with estrogen treatment in maturity-onset diabetes. Diabetes 16: 525.
172. GESSLER, C.J., HALSTED, J.A. & STETSON, R.P. (1939). Effect of estrogenic substances on the blood sugar of female diabetics after the menopause. J. clin. Invest. 18: 715-722.
173. GILMOUR, K.E. & MCKERNS, K.W. (1966). Insulin and estrogen regulation of lipid synthesis in adipose tissue. Biochim. biophys. Acta 116: 220-228.
174. GLEN, A. & EATON, J.C. (1938). Insulin antagonism. Q. Jl. Med. 31: 271-291
175. GOBERNA, R., GARCIA-ALBERTOS, F., TAMARIT-RODRIGUEZ, J., RIO, E. & ROCA, R. (1972). The effects of progesterone and estrogen treatment on insulin secretion after i.v. glucose tolerance tests in normal subjects. In Abstracts of the 8th Annual Meeting of the European Association for the Study of Diabetes. Novo Service. Abstract 106.
176. GOBERNA, R., VOIGT, K.H., FUSSGÄNGER, R.D., LAUBE, H. & PFEIFFER, E.F. (1971). Effect of the synthetic progestin chlormadinone acetate on the insulin response to glucose in normal and subtotaly pancreatectomized rats. Endocrinology 89: 974-980.
177. GOETZ, F.C., GREENBERG, B.Z., ELLIS, J. & MEINERT, C. (1963). A simple immunoassay for insulin: application to human and dog plasma. J. clin. Endocr. Metab. 23: 1237-1246.

178. GOLD, E.M., CARVAJAL, J., RUDNICK, P.A. & GERSZI, K.E. (1969). Insulin production in overt (maturity-onset) diabetes: absence of hyperinsulinemia despite hyperglycemia induced by contraceptive steroids. In Metabolic effects of gonadal hormones and contraceptive steroids, pp. 144-156. New York & London: Plenum Press.
179. GOLDMAN, J.A. & ECKERLING, B. (1970a). Blood glucose levels and glucose tolerance in women with subclinical diabetes receiving an oral contraceptive. Am. J. Obstet. Gynec. 107: 325-327.
180. GOLDMAN, J.A. & ECKERLING, B. (1970b). Blood glucose levels and glucose tolerance in prediabetic and subclinical diabetic women on a low dose progestogen contraceptive. Israel J. med. Sci. 6: 703-707.
181. GOLDMAN, J.A. & ECKERLING, B. (1972). Effect of a progestogen oral contraceptive compound on carbohydrate metabolism. Israel J. med. Sci. 8: 1724-1727.
182. GOLDMAN, J.A., ECKERLING, B. & OVADIA, J.L. (1969). The effect of pseudopregnancy by ovulatory suppressants on the glucose tolerance in women. Fert. Steril. 20: 393-399.
183. GOLDMAN, J.A. & OVADIA, J.L. (1969). The effect of estrogen on intravenous glucose tolerance in women. Am. J. Obstet. Gynec. 103: 172-178.
184. GOLDMAN, J.A., OVADIA, J.L. & ECKERLING, B. (1968). Effect of progesterone on glucose tolerance in women. Israel J. med. Sci. 4: 878-882.
185. GOMMERS, A. (1971). Evolution du coefficient d'assimilation de glucose avec l'âge chez le rat. Gerontologia 17: 228-235.

186. GOMMERS, A. & DE GASPARO, M. (1972). Variation de l'insulinémie en fonction de l'âge chez le rat male non traité. Gerontologia 18: 176-184.
187. GOMORI, G. (1941). Observations with differential stains on human islets of Langerhans. Am. J. Path. 17: 395-406.
188. GOODWIN, J.F. (1970). Method for simultaneous direct estimation of glucose and zylase in serum. Clin. Chem. 16: 85-91
189. GOSPODAROWICZ, D. (1971a). In vivo induction of the effector system for LH in white adipocytes of the rat. Biochem. biophys. Acta 244: 353-359.
190. GOSPODAROWICZ, D. (1971b). Lipolytic activity of the luteinizing hormone (LH) and its two subunits. Endocrinology 89: 571-575.
191. GRAMOVA, E.A., KRAUS, M. & KRECEK, J. (1967). Effect of melatonin and 5-hydroxytryptamine on aldosterone and corticosterone production by adrenal glands of normal and hypophysectomized rats. J. Endocr. 39: 345-350.
192. GRANT, D.B. (1968). Observations on the precipitation reaction in a double-antibody immunoassay for insulin. Acta endocr., Copnh. 59: 139-149.
193. GRANT, D.B. (1972). Serum-insulin or plasma-insulin? Lancet i: 207.
194. GREEN, I.C. & TAYLOR, K.W. (1972). Effects of pregnancy in the rat on the size and insulin secretory response of the islets of Langerhans. J. Endocr. 54: 317-325.
195. GREENE, R. (1958). Phasic insulin resistance associated with the menstrual cycle and controlled by fluoro-hydrocortisone: a case report. Metabolism 7: 90 - 92.

196. GREENWOOD, F.C., HUNTER, W.M. & GLOVER, J.S. (1963).
The preparation of 131 I-labelled human growth hormone of
high specific radioactivity. Biochem. J. 89: 114-123.
197. GRICE, H.C. (1964). Methods for obtaining blood and
for intravenous injections in laboratory animals.
Lab. Anim. Care 14: 483-493.
198. GRIFFITHS, M., MARKS, H.P. & YOUNG, F.G. (1941).
Influence of oestrogens and androgens on glycogen
storage in the fasting rat. Nature, Lond. 147: 359.
199. GRIMELIUS, L. (1964). A modified silver protein method
for studying the argyrophilic cells of the islets of
Langerhans. In The structure and metabolism of the
pancreatic islets, vol. 3, pp. 99-103. Eds. S.E. Brodin,
B. Hellman & H. Knutson. London: Pergamon Press.
200. GRIMELIUS, L. (1968a). A silver nitrate stain for A_2
cells in human pancreatic islets. Acta Soc. Med. upsal.
73: 243-270.
201. GRIMELIUS, L. (1968b). The argyrophil reaction in
islet cells of adult human pancreas studied with a new
silver nitrate procedure. Acta Soc. Med. upsal. 73:
271-294.
202. GRODSKY, G.M. (1970). Insulin and the pancreas.
Vitams Horm. 28: 37-101.
203. GRODSKY, G.M. (1973). A threshold distribution
hypothesis for packet storage of insulin and its
mathematical modeling. J. clin. Invest. 51: 2047-2059.
204. GRODSKY, G.M., BATTS, A.A., BENNETT, L.L., VCELLA, C.,
McWILLIAMS, N.B. & SMITH, D.F. (1963). Effects of
carbohydrates on secretion of insulin from isolated
rat pancreas. Am. J. Physiol. 205: 638-644.

205. GRODSKY, G.M. & FORSHAM, P.H. Immunochemical assay of total extractable insulin in man. J. clin. Invest. 39: 1070-1079.
206. GUSDON, J.P., LEAKE, N.H., VAN DYKE, A.H. & ATKINS, W. (1970). Immunochemical comparison of human placental lactogen and placental proteins from other species. Am. J. Obstet. Gynec. 107: 441-444.
207. HAIST, R.E. (1965). Effects of steroids on the pancreas. Methods Horm. Res. 4: 193-233.
208. HAIST, R.E. & PUGH, E.J. (1948). Volume measurement of the islets of Langerhans and the effects of age and fasting. Am. J. Physiol. 152: 36-41.
209. HAJDU, A., HERR, F. & RONA, G. (1968). The functional significance of a spontaneous pancreatic islet change in aged rats. Diabetologia 4: 44-47.
210. HAJDU, A. & RONA, G. (1967). Morphological observations on spontaneous pancreatic islet changes in rats. Diabetes 16: 108-110.
211. HAJDU, A. & RONA, G. (1971). The protective effect of estrogens against spontaneous pancreatic islet and renal changes in aging male rats. Experientia 27: 956-957.
212. HALES, C.N. (1967). Some actions of hormones in the regulation of glucose metabolism. In Essays in biochemistry, vol.1, pp. 73-104. Eds. P.N. Campbell & G.C. Greville. London and New York: Academic Press.
213. HALES, C.N., GREENWOOD, F.C., MITCHELL, F.L. & STRAUSS, W.T. (1968). Blood-glucose, plasma-insulin and growth hormone concentrations of individuals with minor abnormalities of glucose tolerance. Diabetologia 4: 73-82.

214. HALES, C.N. & MILNER, R.D.G. (1968). The role of sodium and potassium in insulin secretion from rabbit pancreas. J. Physiol., Lond. 194: 725-743.
215. HALES, C.N. & RANDLE, P.J. (1963a). Immunoassay of insulin with insulin-antibody precipitate. Biochem. J. 88: 137-146.
216. HALES, C.N. & RANDLE, P.J. (1963b). Immunoassay of insulin with insulin antibody precipitate. Lancet i: 200.
217. HALLER, J. (1972). Hormonal contraception. Los Altos, California: Geron-X.
218. HALLING, G., MICHALS, E.L. & PAULSEN, C.A. (1967). Glucose intolerance during ethynodiol diacetate-mestranol therapy. Metabolism 16: 465-468.
219. HARROP, G.A. & MOSENTHAL, H.O. (1918). The influence of menstruation on acidosis in diabetes mellitus. Report of a case. Bull. Johns Hopkins Hosp. 29: 161-163.
220. HASHIMOTO, I., HENRICKS, D.M., ANDERSON, L.L. & MELAMPY, R.M. (1968). Progesterone and pregn-4-en-20 α -ol-3-one in ovarian venous blood during various reproductive states in the rat. Endocrinology 82: 333-341.
221. HAYNER, N.S., KJELSBERG, M.O., EPSTEIN, F.H. & FRANCIS, T. (1965). Carbohydrate tolerance and diabetes in a total community, Tecumseh, Michigan. 1. Effects of age, sex, and test conditions on one-hour glucose tolerance in adults. Diabetes 14: 413-423.

222. HEALD, F.P. & HUNG, W. (1970). Endocrine control of adolescent growth spurt. In Adolescent endocrinology, pp. 25-40. Eds. F.P. Heald & W. Hung. London: Butterworths.
223. HEAP, R.B. (1972). Role of hormones in pregnancy. In Hormones in reproduction, vol.3, pp. 73-105. Eds. C.R. Austin & R.V. Short. London: Cambridge University Press.
224. HEBOLD, G. & BLEUEL, H. (1973). Various blood glucose values in the rat. Horm. Metab. Res. 5: 14-17.
225. HEDING, L.G. (1966). A simplified insulin radio-immunoassay method. In Labelled proteins in tracer studies, pp.345-351. Eds. L. Donato, G. Milhand & J. Suchis. Brussels: EURATOM.
226. HEILIG, R. (1924). Menstruationsstudien. I. Zuckerstoffwechsel. Klin. Wschr. 3: 576-577.
227. HELLERSTRÖM, C. (1964). A method for the micro-dissection of intact pancreatic islets of mammals. Acta endocr., Copnh. 45: 122-132.
228. HELLMAN, B. (1959a). The total volume of the pancreatic islet tissue at different ages of the rat. Acta path. microbiol. scand. 47: 35-50.
229. HELLMAN, B. (1959b). The numerical distribution of the islets of Langerhans at different ages of the rat. Acta endocr., Copnh. 32: 63-67.
230. HELLMAN, B. (1959c). The effect of ageing on the number of the islets of Langerhans in the rat. Acta endocr., Copnh. 32: 78-91.

231. HELLMAN, B. (1970). Methodological approaches to studies on the pancreatic islets. Diabetologia 6: 110-120.
232. HELLMAN, B., LERNMARK, Å, SEHLIN, J. & TÄLJEDAL, I. (1972). Transport and storage of 5-hydroxytryptamine in pancreatic B-cells. Biochem. Pharmac. 21: 695-706.
233. HENDERSON, J.R. (1970). Serum-insulin or plasma-insulin? Lancet ii: 545-547.
234. HENDERSON, J.R. (1971). Serum-insulin or plasma-insulin? Lancet ii: 1430-1431.
235. HENRY, R.J. (1966). Clinical chemistry; principles and technics. New York: Hoeber.
236. HERBERT, V., LAU, K., GOTTLIEB, L.W. & BLEICHER, S.J. (1965). Coated charcoal immunoassay of insulin. J. clin. Endocr. Metab. 25: 1375-1384.
237. HERRERA, E. KNOPP, R.H. & FREINKEL, N. (1969). Carbohydrate metabolism in pregnancy. VI. Plasma fuels, insulin, liver composition, gluconeogenesis, and nitrogen metabolism during late gestation in the fed and fasted rat. J. clin. Invest. 48: 2260-2272.
238. HILLMER, T., FRERICHS, H., HALLER, J. & CREUTZFELDT, W. (1968). Kohlenhydrat und fettstoffwechseleränderungen während der Behandlung mit einem ovulationshemmer (Eugynon). Kongress der Deutschen Diabetes-Gesellschaft 3: 11.
239. HINSULL, S.M. (1972). Electrolyte metabolism during the oestrous cycle in the rat. Ph.D. thesis. University of Aston in Birmingham.

240. HINSULL, S.M. & CROCKER, A.D. (1970). The effects of ovarian hormones on the activity of the adrenal cortex and on water and sodium transport. J. Endocr. 48: lxxix-lxxx.
241. HOET, J.P. & LUKENS, F.D.W. (1954). Carbohydrate metabolism during pregnancy. Diabetes 3: 1-12.
242. HOFFMAN, W.S. (1937). A rapid photoelectric method for the determination of glucose in blood and urine. J. biol. Chem. 120: 51-55.
243. HOSHI, M. & SHREEVE, W.W. (1973). Release and production of insulin by isolated, perfused rat pancreatic islets. Diabetes 22: 16-24.
244. HOUSSAY, B.A. (1951). Action of sex hormones on experimental diabetes. Br. med. J. 2: 505-510.
245. HOUSSAY, B.A.; FOGLIA, V.G. & RODRIGUEZ, R.R. (1954). Production or prevention of some types of experimental diabetes by oestrogens and corticosteroids. Acta endocr., Copnh. 17: 146-164.
246. HOWLAND, B.E. & ZEBROWSKI, E.J. (1972). Hyposecretion of gonadotrophins in alloxan-treated male rats. J. Reprod. Fert. 31: 115-118.
247. HUBBLE, D. (1954). Insulin resistance. Br. med. J. 2: 1022-1024.
248. HUERGA, J. & SHERRICK, J.C. (1971). The effect of aging on the glucose tolerance test. In Laboratory diagnosis of endocrine diseases, pp. 390-400. Eds. F.W. Sunderman & F.W. Sunderman jr. London: Adam Hilger.
249. HUFF, T.A., HORTON, E.S. & LEBOVITZ, H.E. (1967). Abnormal insulin secretion in myotonic dystrophy. New Engl. J. Med. 277: 837-841.

250. HUNT, E.L. & BAILEY, D.W. (1961). The effects of alloxan diabetes on the reproductive system of young male rats. Acta endocr., Copnh. 38: 432-440.
251. HURN, B.A.L. & LANDON, J. (1971). Antisera for radioimmunoassay. In Radioimmunoassay methods, pp. 121-142. Eds. K.E. Kirkham & W.M. Hunter. Edinburgh and London: Churchill Livingstone.
252. HURWITZ, A. (1971). A simple method for obtaining blood samples from rats. J. Lab. clin. Med. 78: 172-174.
253. HUTCHERSON, W.P., SCHWARTZ, H.A., WEATHERS, W. & MCGUIRE, J.E. (1967). Long-term evaluation of two oral contraceptives. Fert. Steril. 18: 616-631.
254. IDÄHL, L. (1972). A micro perfusion device for pancreatic islets allowing concomitant recordings of intermediate metabolites and insulin release. Analyt. Biochem. 50: 386-398.
255. INGBAR, S.H. & WOEBAR, K.A. (1969). The thyroid gland. In Textbook of endocrinology, pp. 105-286. Ed. R.H. Williams. Philadelphia, London and Toronto: W.B. Saunders Company.
256. INGLE, D.J. (1941). Diabetogenic effect of stilbestrol in force-fed normal and partially depancreatized rats. Endocrinology 29: 838-848.
257. INGLE, D.J. (1943). The relationship of the diabetogenic effect of diethylstilbestrol to the adrenal cortex in the rat. Am. J. Physiol. 138: 577-582.
258. IRISAWA, S., SHIRAI, M., MATSUSHITA, S., KAGAYAMA, M. & ICHIJO, S. (1966). Sexual disturbances in diabetes. Tohoku J. exp. Med. 88: 311-326.

259. IZZO, J.L., BALE, W.F., IZZO, M.J. & RONCONE, A. (1964). High specific activity labelling of insulin with ^{131}I . J. biol. Chem. 239: 3743-3748.
260. JACOBS, P. & ADRIAENSSEAS, L. (1970). A simple method for repeated blood sampling in small animals. J. Lab. clin. Med. 75: 1013-1016.
261. JAMES, F. & FOTHERBY, K. (1970). Interaction of sex hormones with target tissues. In Advances in steroid biochemistry and pharmacology, vol. 2, pp. 315-472. Ed. M.H. Briggs. London and New York: Academic Press.
262. JARRETT, R.J. & GRAVER, H.J. (1968). Changes in oral glucose tolerance during the menstrual cycle. Br. med. J. 2: 528-529.
263. JAVIER, Z., GERSHBERG, H. & HULSE, M. (1968). Ovulating suppressants, estrogens and carbohydrate metabolism. Metabolism 17: 443-456.
264. JENSEN, E.V. & DESOMBRE, E.R. (1972). Mechanism of action of the female sex hormones. A. Rev. Biochem. 41: 203-230.
265. JOHANSEN, K. (1972). Normal initial insulin response in elderly people with glucose tolerance test diabetes. Israel J. med. Sci. 8: 918.
266. JOLIN, T. & MONTES, A. (1973). Daily rhythm of plasma glucose and insulin levels in rats. Hormone Res. 4: 153-156.
267. JONES, V.E. & CUNLIFFE, A.C. (1961). A precipitating antibody to insulin. Nature, Lond. 192: 136-138.
268. JORGENSEN, K.R. (1966). Immunoassay of insulin in human urine. Acta endocr., Copnh. 51: 400-410.

269. JUNOD, A., LETARTE, J., LAMBERT, A.E. & STAUFFACHER, W. (1969). Studies in spiny mice (Acomys Cahirinus): metabolic state and pancreatic insulin release in vitro. Horm. Metab. Res. 1: 45-52.
270. KADISH, A.H., LITTLE, R.C. & STERNBERG, J.C. (1968). A new and rapid method for the determination of glucose by measurement of rate of oxygen consumption. Clin. Chem. 14: 116-131.
271. KALKHOFF, R.K. (1972). Effects of oral contraceptive agents and sex steroids on carbohydrate metabolism. A. Rev. Med. 23: 429-438.
272. KALKHOFF, R.K., JACOBSON, M. & LEMPER, D. (1970). Progesterone, pregnancy and the augmented plasma insulin response. J. clin. Endocr. Metab. 31: 24-28.
273. KALKHOFF, R.K., KIM, H.J. & STODDARD, F.J. (1968). Acquired subclinical diabetes mellitus in women receiving oral contraceptive agents. Diabetes 17: (suppl.1), 307.
274. KALKHOFF, R.K., KIM, H.J. & STODDARD, F.J. (1969). Acquired subclinical diabetes mellitus in women receiving oral contraceptive agents. In Metabolic effects of gonadal hormones and contraceptive steroids, pp. 193-203. Eds. H.A. Salhanick, D.M. Kipnis & R.L. Vande Wiele. New York & London: Plenum Press.
275. KALKHOFF, R.K., RICHARDSON, B.L. & BECK, P. (1969). Relative effects of pregnancy, human placental lactogen and prednisolone on carbohydrate tolerance in normal and subclinical diabetic subjects. Diabetes 18: 153-175.
276. KALKHOFF, R.K., SCHALCH, D.S., WALKER, J.L., BECK, P., KIPNIS, D.M. & DAUGHADAY, W.H. (1964). Diabetogenic factors associated with pregnancy. Trans. Ass. Am. Physns. 77: 270-280.

277. KAMBERI, I.A., MICAL, R.S. & PORTER, J.C. (1971).
Effect of melatonin and serotonin on the release of
FSH and prolactin. Endocrinology 88: 1288-1293.
278. KANAZAWA, Y., KUZUYA, T., IDE, T. & KOSAKA, K. (1966).
Plasma insulin responses to glucose in femoral,
hepatic and pancreatic veins in dogs. Am. J. Physiol.
211: 442-448.
279. KASSELL, B. (1970). Naturally occurring inhibitors of
proteolytic enzymes. Meth. Enzym. 19: 839-906.
280. KEEN, H., SELLS, R. & JARRETT, R.J. (1965). A method
for the study of the metabolism of isolated mammalian
islets of Langerhans and some preliminary results.
Diabetologia 1: 28-32.
281. KENT, J.R. (1966). Gonadal function in impotent
diabetic males. Diabetes 15: 537.
282. KERP, L., KASEMIUR, H. & STEINHILBER, S. (1971).
Sources of error in the insulin immunoassay due to
fragments of bovine insulin and bovine proinsulin.
Horm. Metab. Res. Suppl. 3: 29-32.
283. KERR, E.H., STEARS, J.C., MacDOUGALL, I. & HAIST, R.E.
(1952). Influence of gonads on growth of islets of
Langerhans. Am. J. Physiol. 170: 448-455.
284. KIM, J.N., RUNGE, W., WELLS, L.J. & LAZAROW, A. (1960).
Effects of experimental diabetes on the offspring of
the rat. Fetal growth, birth weight, gestation period
and fetal mortality. Diabetes 9: 396-404.
285. KINSELL, L.W., MARGEN, S. & MICHAELS, G.D. (1949).
A consideration of the effect of testosterone propionate
upon ketone metabolism. Proc. Am. Diabetes Ass. 9:
123-132.

286. KITAO, M. (1971). Sex hormones and lipid metabolism. Yonago Acta medica 15: 138-144.
287. KITAY, J.I. (1963). Effects of estradiol on pituitary-adrenal function in male and female rats. Endocrinology 72: 947-953.
288. KLEBANOW, D. & MacLEOD, J. (1960). Semen quality and certain disturbances of reproduction in diabetic men. Fert. Steril. 11: 255-261.
289. KLIMAS, J.E. (1968). Oral glucose tolerance during the life-span of a colony of rats. J. Geront. 23: 31-34.
290. KNOPP, R.H., RUDER, H.J., HERRERA, E. & FREINKEL, N. (1970). Carbohydrate metabolism in pregnancy. VII. Insulin tolerance during late pregnancy in the fed and fasted rat. Acta endocr., Copnh. 65: 352-360.
291. KOBBERLING, J. & CREUTZFELDT, W. (1970). Comparison of different methods for the evaluation of the oral glucose tolerance test. Diabetes 18: 870-877.
292. KOLODNY, R.C. (1971). Sexual dysfunction in diabetic females. Diabetes 20: 557-559.
293. KOPIN, I.J., PARE, C.M.B., AXELROD, J. & WEISSBACH, H. (1961). The fate of melatonin in animals. J. biol. Chem. 236: 3072-3075.
294. KOSTIANOVSKY, M., LACY, P.E., GREIDER, H.M. & STILL, M.F. (1972). Long term (15 days) incubation of islets of Langerhans isolated from adult rats and mice. Lab. Invest. 27: 53-61.
295. KYLE, G.C. (1963). Diabetes and pregnancy. Ann. intern. Med. 59: (suppl.3), 1-82.

296. LABHSETWAR, A.P. (1972). Peripheral serum levels of immunoreactive 'oestradiol' in rats during various reproductive states: adrenal contribution of immunoreactive material. J. Endocr. 52: 399-400.
297. LACY, P.E. & KOSTIANOVSKY, M. (1967). Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 16: 35-39.
298. LACY, P.E., WALKER, M.M. & FINK, C.J. (1972). Perifusion of isolated rat islets in vitro. Diabetes 21: 987-998.
299. La HABA, A.F., RODRIGUEZ, G.V. & PELLEGRINA, I. (1971). Carbohydrate metabolism in long-term oral contraceptive users. Obstet. Gynec. 37: 220-224.
300. LAMBERT, A.E., BLONDEL, B., KANAZAWA, Y., ORCI, L. & RENOLD, A.E. (1972). Monolayer culture of neonatal rat pancreas: light microscopy and evidence of immunoreactive insulin synthesis and release. Endocrinology 90: 239-248.
301. LANDGRAF, R., KOTLER-BRAJTBURG, J. & MATSCHINSKY, F.M. (1971). Kinetics of insulin release from the perfused rat pancreas caused by glucose, glucosamine, and galactose. Proc. Natn. Acad. Sci. U.S.A. 68: 536-540.
302. LARSSON-COHN, U., TENGSTRÖM, B. & WIDE, L. (1969). Glucose tolerance and insulin response during daily continuous low-dose oral contraceptive treatment. Acta endocr., Copnh. 62: 242-250.
303. LAWRENCE, A.M. & CONTOPOULOS, A.N. (1960). Reproductive performance in the alloxan diabetic female rat. Acta endocr., Copnh. 33: 175-184.

304. LEADING ARTICLE (1970). Diabetes and menstruation. Br. med. J. 4: 699.
305. LEI, K.Y. & YANG, M.G. (1972). Oral contraceptives, norethynodrel and mestranol: effects on glucose tolerance, tissue uptake of glucose-U-¹⁴C and insulin sensitivity. Proc. Soc. exp. Biol. Med. 141: 130-136.
306. LEONARD, S. (1952). Effect of castration and testosterone propionate injection on glycogen storage in skeletal muscle. Endocrinology 51: 293-297.
307. LEWIS, J.T., FOGLIA, V.G. & RODRIGUEZ, R.R. (1950). The effects of steroids on the incidence of diabetes in rats after subtotal pancreatectomy. Endocrinology 46: 111-121.
308. LIDDLE, G.W. (1969). Summarization of the effects of hormones on water and electrolyte metabolism. In Textbook of endocrinology, pp. 1119-1129. Ed. R.H. Williams. Philadelphia, London & Toronto: W.B. Saunders Company.
309. LIN, T.J., LIN, S.C., ERLÉNMEYER, F., KLINE, I.T., UNDERWOOD, R., BILLIAR, R.B. & LITTLE, B. (1972). Progesterone production rates during the third trimester of pregnancy in normal women, diabetic women, and women with abnormal glucose tolerance. J. clin. Endocr. Metab. 34: 287-297.
310. LIN, T.J., LIN, S.C. & JOHNSON, D.C. (1972). Serum FSH, LH and the ovarian response to exogenous gonadotrophins in alloxan diabetic immature female rats. Endocrinology 91: 1172-1179.
311. LINDAN, O. & MORGAN, M.E. (1950). Alloxan diabetes and pregnancy: a long term observation. J. Endocr. 6: 463-469.

312. LOGAN EDWARDS, R. (1973). Personal communication.
313. LOGSDON, D.F. (1971). Comparison of the two-antibody and charcoal radioimmunoassays for human plasma insulin. Radiol. Technol. 42: 387-391.
314. LONG, J.A. (1946). Pulsating perfusing apparatus. Science, N.Y. 103: 170.
315. LONG, J.A. & EVANS, H.M. (1922). The oestrous cycle in the rat and its associated phenomena. Mems. Univ. Calif. 6: 1-148.
316. LOUBATIÈRES, A., MARIANI, M.M. & CHAPAL, J. (1968). Inhibition de la secretion d'insuline par l'elevation de la teneur en insuline du milieu irriguant les cellules beta des ilots de Langerhans du pancreas. C. r. hebd. Séanc. Acad. Sci., Paris 266: 2245-2247.
317. LOVE, E.J., STEVENSON, J.A.F. & KINCH, R.A.H. (1964). Evaluation of oral and intravenous glucose tolerance tests for the diagnosis of "prediabetes" in the puerperium. Am. J. Obstet. Gynec. 88: 283-290.
318. LOVLESS, M.H. (1956). A means of estimating circulating insulin in man. Q. Rev. Allergy appl. Immun. 10: 374-385.
319. LUNDQUIST, I. (1971). Insulin secretion: its regulation by monoamines and acid amyloglucosidase. Acta physiol. scand. suppl. 372: 1-47.
320. LUNDQUIST, I. (1972a). Acid amyloglucosidase and carbohydrate regulation. 1. Effect of exogenous amyloglucosidase on tissue glycogen, blood glucose and plasma insulin. Horm. Metab. Res. 4: 151-158.

321. LUNDQUIST, I. (1972b). Acid amyloglucosidase and carbohydrate regulation. II. Acid amyloglucosidase activity in the endocrine pancreas. Horm. Metab. Res. 4: 245-249.
322. LUNDQUIST, I. (1972c). Acid amyloglucosidase and carbohydrate regulation. III. The induction of sulphonylurea-stimulated insulin release and its dependence on intracellular monoamines. Horm. Metab. Res. 4: 341-348.
323. LUNNELL, NO. & PERSSON, B. (1971). The influence of oral contraceptives on carbohydrate and lipid metabolism. In Current problems in fertility, pp. 170-180. Ed. A. Ingelman-Sundberg. New York & London: Plenum Press.
324. MACCHI, I.A. & BLAUSTEIN, E.H. (1969). Cytostructure and endocrine function of monolayer cultures of neonatal hamster pancreas. Endocrinology 84: 208-216.
325. MACDONALD, H.M., GOOD, W., SCHWARTZ, K. & STONE, J. (1971a). Serial observations of glucose tolerance in pregnancy and the early puerperium. J. Obstet. Gynaec. Br. Commonw. 78: 489-497.
326. MACDONALD, H.N., GOOD, W., SCHWARZ, K. & STONE, J. (1971b). Oral glucose tolerance in the early puerperium. J. Obstet. Gynaec. Br. Commonw. 78: 1004-1006.
327. MACDONALD, I. & CROSSLEY, J.N. (1970). Glucose tolerance during the menstrual cycle. Diabetes 19: 450-452.
328. MAJOR, P.W. & KILPATRICK, R. (1972). CyclicAMP and hormone action. J. Endocr. 52: 593-630.

329. MAKULU, D.R., VICHICK, D., WRIGHT, P.H., SUSSMAN, K.E. & YU, P. (1969). Insulin immunoassay by back-titration using alcohol precipitation of insulin-antibody complexes. Diabetes 18: 660-669.
330. MALAISSE, W.J. (1972). Hormonal and environmental modification of islet activity. In Handbook of physiology, section 7, vol.1, pp 237-260. Eds R.O. Greep and E.B. Astwood. Washington, D.C.: American Physiological Society.
331. MALAISSE, W.J., LAUVAUX, J.P., FRANCKSON, J.R.M. & BASTENIE, P.A. (1965). Diabetes in bearded women (Achard-Thiers-syndrome). Diabetologia 1: 155-161.
332. MALAISSE, W.J., MALAISSE-LAGAE, F., LACY, P.E. & WRIGHT, P.H. (1967a). Insulin secretion by isolated islets in presence of glucose, insulin and anti-insulin serum. Proc. Soc. exp. Biol. Med. 124: 497-500.
333. MALAISSE, W.J., MALAISSE-LAGAE, F. & MAYHEW, D. (1967b). A possible role for the adenylcyclase system in insulin secretion. J. clin. Invest. 46: 1724-1734.
334. MALAISSE, W.J., MALAISSE-LAGAE, F. & WRIGHT, P.H. (1967c). A new method for the measurement in vitro of pancreatic insulin secretion. Endocrinology 80: 99-108.
335. MALAISSE, W.J., MALAISSE-LAGAE, F., LACY, P.E. & WRIGHT, P.H. (1967d). Insulin secretion by isolated islets in presence of glucose, insulin and anti-insulin serum. Proc. Soc. exp. Biol. Med. 124: 497-500.
336. MALAISSE, W.J., MALAISSE-LAGAE, F., PICARD, C. & FLAMENT-DURAND, J. (1969). Effects of pregnancy and chorionic growth hormone upon insulin secretion. Endocrinology 84: 41-44.

337. MALINS, J. (1968). Clinical diabetes mellitus.
London: Eyre & Spottiswoode Ltd.
338. MALINS, J.M., FITZGERALD, M.G. & WALL, M. (1965).
A change in the sex incidence of diabetes mellitus.
Diabetologia 1: 121-124.
339. MARCUS, H. & GLOTZER, S. (1948). Estrogens in
diabetes. N.Y. St. J. Med. 48: 1461-1464.
340. MARIGO, S. (1968). The insulin secretion in old age.
Acta Diab. Latina 5: (suppl.1), 359-369.
341. MARINE, N., VINIK, A.I., EDELSTEIN, I. & JACKSON,
W.P.U. (1969). Diabetes, hyperglycemia and glycosuria
among Indians, Malays and Africans (Bantu) in Cape
Town, South Africa. Diabetes 18: 840-857.
342. MARKUSSEN, J., HEDING, L.G., JORGENSEN, K.H. &
SUNDBY, F. (1971). Proinsulin, insulin and c-peptide.
Horm. Metab. Res. Suppl. 3: 33-35.
343. MATUTE, M.L. & KALKHOFF, R.K. (1973). Sex steroid
influence on hepatic gluconeogenesis and glycogen
formation. Endocrinology 92: 762-768.
344. MAYHEW, D.A., WRIGHT, P.H. & ASHMORE, J. (1969).
Regulation of insulin secretion. Pharmac. Rev. 21:
183-212.
345. McCULLUGH, E.P., BECK, J.C. & SCHAFFENBERG, C.A.
(1955). Control of diabetes and other features of
acromegaly following treatment with estrogens.
Diabetes 4: 13-23.
346. McGUIRE, J.L. & LISK, R.D. (1968). Estrogen receptors
in the intact rat. Proc. natn. Acad. Sci. U.S.A. 61:
497-503.

347. MCFARLANE, A.S. (1958). Efficient trace-labelling of proteins with iodine. Nature, Lond. 182: 53.
348. MCGAVACK, T.H. (1967). Endocrine ichnography of aging. In Endocrines and aging, pp. 36-50. Ed. L. Gitman. Springfield, Illinois: Charles C. Thomas.
349. McKERNS, K.W. (Ed.) (1971). The sex steroids. Molecular mechanisms. New York: Meredith Corporation.
350. MEADE, R.C. & KLITGAARD, H.M. (1962). A simplified method for the immunoassay of human serum insulin. J. nucl. Med. 3: 407-416.
351. MEANS, A.R. & O'MALLEY, B.W. (1972). Mechanism of estrogen action: early transcriptional and translational events. Metabolism 21: 357-370.
352. MEITES, S. & SANIEL-BANREY, K. (1973). Modified glucose oxidase method for determination of glucose in whole blood. Clin. Chem. 19: 308-311.
353. MELLONI, G.F. & PECCHIAI, L. (1957). Modifications histologiques et histochimiques du pancreas insulaire sous l'influence des stimulines hypophysaires. Annls Endocr. 18: 601-603.
354. MESTMAN, J.H., ANDERSON, G.V. & BARTON, P. (1971). Carbohydrate metabolism in pregnancy. Am. J. Obstet. Gynec. 109: 41-45.
355. METCALF, M. & BEAVEN, D.W. (1963). Plasma corticosteroid levels in women receiving oral contraceptive tablets. Lancet 2: 1095-1096.
356. METROPOLITAN LIFE INSURANCE COMPANY (1970). Diabetes at midlife. Statist. Bull. Metrop. Life Insur. Co. 51: 2-4

357. METROPOLITAN LIFE INSURANCE COMPANY (1972). Diabetes mortality - United States, Canada, and Western Europe. Statist. Bull. Metrop. Life Insur. Co. 53: 5-8.
358. MIAHLE, P. & MEYER, V. (1961). Secretion d'insuline par le pancreas de rat in vitro. C. r. Séanc. Soc. Biol. 253: 1861-1863.
359. MIDGLEY, A.R., NISWENDER, G.C. & REGAR, R.W. (1969). Principles for the assessment of the reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity). Acta endocr., Copnh. 63: Suppl. 142, 163-184.
360. MIEDEMA, K., BOELHOUWER, J. & OTTEN, J.W. (1972). Determinations of proteins and hormones in serum by an immunoassay using antigen-enzyme conjugates. Clinica chim. Acta 40: 187-192.
361. MILCU, S.M., NANU, L. & MILCU, I. (1971). The effect of pinealectomy on plasma insulin in rats. In The pineal gland, pp. 345-360. Eds G.E.W. Wolstenholme & J. Knight. London: Churchill Livingstone.
362. MILES, L.E.M. (1971). Noncompetitive binding assays using labelled antibodies. In Principles of competitive protein-binding assays, pp. 260-281. Eds. W.D. Odell & W.H. Daughaday. Philadelphia and Toronto: J.B. Lippincott Company.
363. MILES, L.E.M. & HALES, C.N. (1968a). The preparation and properties of purified ¹²⁵I-labelled antibodies to insulin. Biochem. J. 108: 611-618.
364. MILES, L.E.M. & HALES, C.N. (1968b). Labelled antibodies and immunological assay systems. Nature, Lond. 219: 186-189.

365. MINTZ, D.D., FINSTER, J.L. & JOSIMOVICH, J.B. (1967). Effect of estrogen therapy on carbohydrate metabolism in acromegaly. J. clin. Endocr. Metab. 27: 1321-1327.
366. MITCHELL, M.L. & BYRON, J. (1967). Use of enzyme proteolysis for the immunochemical measurement of insulin. Diabetes 16: 656-663.
367. MOGHISSI, K.S., SYNER, F.N. & EVANS, T.N. (1972). A composite picture of the menstrual cycle. Am. J. Obstet. Gynec. 114: 405-418.
368. MOLONEY, P.J. & APRILE, M.A. (1959). On the antigenicity of insulin: flocculation of insulin-antiinsulin. Can. J. Biochem. 37: 793-800.
369. MONTAGUE, W. & HOWELL, S.L. (1972). The mode of action of adenosine 3':5'-cyclic monophosphate in mammalian islets of Langerhans. Biochem. J. 129: 551-560.
370. MONROE, S.E., REBAR, R.W., GAY, U.L. & MIDGLEY, A.R. (1969). Radioimmunoassay determination of luteinizing hormone during the estrous cycle of the rat. Endocrinology 85: 720-724.
371. MORGAN, C.R. & LAZAROW, A. (1963). Immunoassay of insulin: two antibody system. Plasma insulin levels in normal, subdiabetic and diabetic rats. Diabetes 12: 115-126.
372. MORGAN, C.R., SORENSON, R.L. & LAZAROW, A. (1964). Studies of an inhibitor of the two antibody immunoassay system. Diabetes 13: 1-5.
373. MORTON, J.H. & MCGAVACK, T.H. (1946). The influence of ovarian activity and administered estrogens upon diabetes mellitus: case report. Ann. int. Med. 25: 154-161.

374. MOSES, L.E. & GOLDZIEHER, J.W. (1970). The influence of a sequential oral contraceptive on the carbohydrate metabolism of diabetics and prediabetics. In Advances in Planned Parenthood, vol.4, pp.101-104. Amsterdam: Excerpta Medica Foundation.
375. MOSKALEWSKI, S. (1965). Isolation and culture of the islets of Langerhans of the guinea pig. Gen. comp. Endocr. 5: 342-353.
376. MUELLER, G.C., VONDERHAAR, B., KIM, U.H., & LE MAHIEU, M. (1972). Estrogen action: an inroad to cell biology. Recent Prog. Horm. Res. 28: 1-49.
377. NATELSON, S. (1971). Techniques of clinical chemistry, 3rd edn. Springfield, Illinois: Charles C. Thomas.
378. NELSON, D.H. (1969). Adrenocortical function related to use of oral contraceptives. J. Reprod. Med. 3: 17-19.
379. NICHOLSON, W.A. (1971). Changing sex ratio in diabetes. Br. med. J. 4: 465-466.
380. NICOL, D.S.H.W. (1960). The biological activity of pure peptides obtained by enzymic hydrolysis of insulin. Biochem. J. 75: 395-401.
381. NIELSEN, F.H. (1972). Intravenous glucose tolerance, insulin response, fasting blood glucose and serum insulin during short-term administration of a combined oral contraceptive. Acta Obstet. Gynec. Scand. 51: 319-324.
382. OAKLEY, W.G. & PEEL, Sir J. (1968). Pregnancy. In Clinical diabetes and its biochemical basis, pp. 624-662. W.G. Oakley, D.A. Pyke & K.W. Taylor. Oxford and Edinburgh: Blackwell Scientific Publications.

383. OAKLEY, W.G., PYKE & D.A. & TAYLOR, K.W. (1968).
Clinical diabetes and its biochemical basis. Oxford
and Edinburgh: Blackwell Scientific Publications.
384. O'CONNOR, K.J. (1973). Personal communication, The
Wellcome Foundation Ltd., Dartford.
385. OKEY, R. & ROBB, E.I. (1925). Studies of the
metabolism of women. I. Variations in the fasting
blood sugar level and in sugar tolerance in relation
to the menstrual cycle. J. biol. Chem. 65: 165-186.
386. OLEFSKY, J.M., FARQUHAR, J.W. & REAVEN, G.M. (1973).
Do the oral and intravenous glucose tolerance tests
provide similar diagnostic information in patients with
chemical diabetes mellitus. Diabetes 22: 202-209.
387. O'MALLEY, B.W. (1971). Unified hypothesis for early
biochemical sequence of events in steroid hormone
action. Metabolism 20: 981-988.
388. OOMS, H.A. & ARQUILLA, E.R. (1966). Influence of
iodination on the immunologic properties of insulin.
In Labelled proteins in tracer studies, pp. 237-245.
Eds. L. Donato, G. Milhaud & J. Sirchis. Brussels:
EURATOM.
389. OROSZ, L. (1973). Serum insulin or plasma-insulin?
Lancet i: 491-492.
390. OROSZ, L., MICHAEL, R. & ZIEGLER, M. (1971). Serum-
insulin or plasma-insulin? Lancet ii: 1149-1150.
391. ORSETTI, A. & BOURGEOIS, J.M. (1971). Effets compares
de trois progestatifs sur l'insulinemie chez la chienne.
C. r. Séanc. Soc. Biol. 169: 1406-1410.
392. ORSKOV, H. (1967). Wick-chromatography for the
immunoassay of insulin. Scand. J. clin. Lab. Invest.
20: 297-304.

393. ORSKOV, H. & JOHANSEN, K. (1972). Immunological measurement of urinary insulin for the evaluation of insulin production. Acta endocr., Copnh. 71: 697-708.
394. O'SULLIVAN, J.B. & MAHAN, C.M. (1973). Diagnostic capabilities of the intravenous glucose tolerance test. J. Chron. Dis. 26: 153-161.
395. O'SULLIVAN, J.B., MAHAN, C.M., FREEDLENDER, A.E. & WILLIAMS, R.F. (1971). Effect of age on carbohydrate metabolism. J. clin. Endocr. Metab. 33: 619-623.
396. O'SULLIVAN, J.B., SNYDER, P.J., SPORER, A.C., DANDROW, R.V. & CHARLES, D. (1970). Intravenous glucose tolerance test and its modification by pregnancy. J. clin. Endocr. Metab. 31: 33-37.
397. OUCHTERLONY, O. (1948). In vitro method for testing the toxin-producing capacity of diphtheria bacteria. Acta path. microbiol. scand. 25: 186-191.
398. PALMIERI, G.M.A., YALOW, R.S. & BERSON, S.A. (1971). Adsorbent techniques for the separation of antibody-bound from free peptide hormones in radioimmunoassay. Horm. Metab. Res. 3: 301-305.
399. PAPANICOLAOU, A.D., LORAIN, J.A. & DOVE, G.A. (1969). Endocrine function in postmenopausal women. J. Obstet. Gynaec. Br. Commonw. 76: 317-322.
400. PAUL, P.K. (1971). Changes in hepatic and uterine glycogen following chronic estrogen treatment in the rat. Endokrinologie 58: 87-92.
401. PAUL, P.K. & DUTTAGUPTA, P.N. (1973). Inhibition of oestrogen induced increase in hepatic and uterine glycogen by progesterone in the rat. Acta endocr., Copnh. 72: 762-770.

402. PEEL, Sir J. (1972). A historical review of diabetes and pregnancy. J. Obstet. Gynaec. Br. Commonw. 79: 385-395.
403. PEHRSON, S.L. (1970). Oral contraceptives and glucose tolerance. Acta Obstet. Gynec. Scand. 49: 249-254.
404. PELLEGRINI, G. (1941). Studi sassar. 19: 223.
(Cited by Talaat et al., 1957).
405. PENG, M.-T. & HUANG, H.-H. (1972). Aging of hypothalamic-pituitary-ovarian function in the rat. Fert. Steril. 23: 535-542.
406. PEPERKORN, R. (1932). Menstruation und diabetes mellitus. Munch. med. Wschr. 79: 1748-1753.
407. PETERSON, W.F., STEEL, M.W. & COYNE, R.V. (1966). Analysis of the effects of ovulatory suppressants on glucose tolerance. Am. J. Obstet. Gynec. 95: 484-488.
408. PETROW, V. (1971). Antifertility agents. Prog. Med. Chem. 8: 171-229.
409. PHILLIPS, N. & DUFFY, T. (1973). One-hour glucose tolerance in relation to the use of contraceptive drugs. Am. J. Obstet. Gynec. 116: 91-100.
410. PI-SUNYER, F.X. & OSTER, S. (1968). Effect of an ovulatory suppressant on glucose tolerance and insulin secretion. Obstet. Gynec. 31: 482-484.
411. PLANAS, J., MARTIN-MATEO, M.C. & LAGUNA, M. (1967). Influencia de las hormonas sexuales en la curva de tolerancia de glucosa. R. esp. Fisiol. 23: 155-160.

412. POFFENBARGER, P.L., ESPINOSA DE LOS MONTERO MENA, A. & STEINKE, J. (1970). Nonsuppressible insulin-like activity: immunochemical and physiochemical evidence against its pancreatic origin. Metabolism 19: 509-517.
413. POND, H. & OAKLEY, W.G. (1968). Diabetes in children. In Clinical diabetes and its biochemical basis, pp. 590-623. W.G. Oakley, D.A. Pyke & K.W. Taylor. Oxford and Edinburgh: Blackwell Scientific Publications.
414. POPOVIC, V. & POPOVIC, P. (1960). Permanent cannulation of aorta and vena cava in rats and ground squirrels. J. appl. Physiol. 15: 727-728.
415. PORTE, D. (1969). Sympathetic regulation of insulin secretion. Archs intern. Med. 123: 252-260.
416. PORTE, D. & BAGDADE, J.D. (1970). Human insulin secretion: an integrated approach. A. Rev. Med. 21: 219-240.
417. PORTER, G. (1967). The Norway rat. In The U F A W handbook on the care and management of laboratory animals, 3rd edn, pp. 353-390. Eds. The staff of U F A W. Edinburgh and London: E. & S. Livingstone Ltd.
418. POSNER, N.A., SILVERSTONE, F.A., POMERANCE, W. & BAUMGOLD, D. (1967a). Oral contraceptives and intravenous glucose tolerance. I. Data noted early in treatment. Obstet. Gynec. 29: 79-86.
419. POSNER, N.A., SILVERSTONE, F.A., POMERANCE, W. & SINGER, N. (1967b). Oral contraceptives and intravenous glucose tolerance. II. Long term effect. Obstet. Gynec. 29: 87-92.

420. PUCHULU, J.F., Di PAOLA, G., MARTI, M.L., ROBIN, M., NICHOLSON, R. & GROPPA, S. (1967). Oral contraceptives and diabetes. In Abstracts of the 6th Congress of the International Diabetes Federation, p. 122. Int. Congr. Series no. 140. Amsterdam: Excerpta Medica Foundation.
421. PYKE, D.A. (1968). Aetiological factors. In Clinical diabetes and its biochemical basis, pp. 210-251. W.G. Oakley, D.A. Pyke & K.W. Taylor. Oxford and Edinburgh: Blackwell Scientific Publications.
422. PYORALA, K. PYORALA, T. & LAMPINEN, V. (1967). Sequential oral contraceptive treatment and intravenous glucose tolerance. Lancet 2: 766-777.
423. PYORALA, K., PYORALA, T., LAMPINEN, V. & TASKINEN, M.R. (1970). Glucose tolerance, plasma insulin and lipids in postmenopausal women during sequential oestrogen-progestin treatment. Diabetologia 6: 659.
424. PYORALA, K., PYORALA, T., LAMPINEN, V. & TASKINEN, M.R. (1971). Glucose tolerance, plasma insulin and lipids in postmenopausal women during sequential oestrogen-progestin treatment. Acta Obstet. Gynec. Scand. 50: (suppl.9), 28.
425. PYORALA, K., PYORALA, T. & WEINTRAUB, C. (1971). Glucose tolerance, plasma insulin and lipids in postmenopausal women during oestradiol valerate treatment. Diabetologia 7: 402-403.
426. QUABBE, H.-J. (1969). Modifikation der radioimmunologischen Insulinbestimmung nach Hales und Randle. Diabetologia 5: 101-107.
427. QUICKEL, K.E., FELDMAN, J.M. & LEBOVITZ, H.E. (1971a). Inhibition of insulin secretion by serotonin and dopamine: species variation. Endocrinology 89: 1295-1302.

428. QUICKEL, K.E., FELDMAN, J.M. & LEBOVITZ, H.E. (1971b). Enhancement of insulin secretion in adult onset diabetes by methysergide maleate: evidence for an endogenous biogenic monoamine mechanism as a factor in the impaired insulin secretion in diabetes mellitus. J. clin. Endocr. Metab. 33: 877-881.
429. RABINOWITZ, D. (197). Some endocrine and metabolic aspects of obesity. A. Rev. Med. 21: 241-258.
430. RADIOCHEMICAL CENTRE (1969). Insulin immunoassay kit instruction leaflet. The Radiochemical Centre, Amersham.
431. RADIOCHEMICAL CENTRE (1973). Personal communication: Consumer Service Unit, The Radiochemical Centre, Amersham.
432. RANDLE, P.J. (1970). Blood glucose homeostasis - glucose utilization. In Pathogenesis of diabetes mellitus, pp. 173-197. Eds E. Cerasi & R. Luft. New York, London and Sydney: John Wiley & Sons, Inc.
433. RANDLE, P.J., ASHGROFT, S.J.H. & GILL, J.R. (1968). Carbohydrate metabolism and release of hormones. In Carbohydrate metabolism and its disorders, vol.1 pp. 427-447. Eds F. Dickens, P.J. Randle & W.J. Whelan. London and New York: Academic Press.
434. RANDLE, P.J. & HALES, C.N. (1972). Insulin release mechanism. In Handbook of physiology, section 7, vol.1, pp. 219-236. Eds R.O. Greep & E.B. Astwood. Washington, D.C.: American Physiological Society.
435. RANDLE, P.J. & TAYLOR, K.W. (1960). Insulin in blood. Br. med. Bull. 16: 209-213.

436. RAPS, D., BARTHE, P.L. & DESAULLES, P.A. (1971). Plasma and adrenal corticosterone levels during the different phases of the sexual cycle in normal female rats. Experientia 27: 339-340.
437. RAPTIS, S. (1971). Radioimmunoassay of insulin: comparison of different separation techniques. Horm. Metab. Res. Suppl. 3: 26-28.
438. RAVIN, H.A., BERNSTEIN, P. & SELIGMAN, A.M. (1954). A colorimetric micromethod for the estimation of chymotrypsin activity. J. biol. Chem. 208: 1-15.
439. REINKE, U., ANSAH, B. & VOIGT, K.D. (1972). Effect of the menstrual cycle on carbohydrate and lipid metabolism in normal females. Acta endocr., Copnh. 69: 762-768.
440. REPORT OF THE COMMITTEE ON STATISTICS OF THE AMERICAN DIABETES ASSOCIATION (1969). Standardization of the oral glucose tolerance test. Diabetes 18: 299-307.
441. RICE-WRAY, E., de FERRIER, S.A., PEREZ-HUERTA, I. & GORODOVSKY, J. (1970). Clinical evaluation of a new combined oral contraceptive. Contraception 1: 389-402.
442. RISHI, S., GOLOB, E.K., BECKER, K.L. & SHAH, N. (1969). Pancreatic insulin content of nonpregnant, pregnant and postpartum rats and the developing rat fetus. Diabetes 18: 268-272.
443. ROBISON, G.A., BUTCHER, R.W. & SUTHERLAND, E.W. (1971). Cyclic AMP. New York: Academic Press.
444. RODRIGUEZ, R.R. (1965). Influence of estrogens and androgens on the production and prevention of diabetes. In On the nature and treatment of diabetes, pp. 288-307. Eds. B.S. Leibel & G.A. Wrenshall. Amsterdam: Excerpta Medica Foundation.

445. ROECKEL, I.E. (1971). Tolerance tests for evaluation of carbohydrate metabolism. In Laboratory diagnosis of endocrine diseases, pp. 385-389. Eds. F.W. Sunderman & F.W. Sunderman, jr. London: Adam Hilger.
446. ROSENBLOOM, A.L. & ALLEN, C.M. (1973). Screening of glucose intolerance in a pediatric multiphasic program. Metabolism 22: 319-322.
447. ROSENBLOOM, J. (1921). Influence of menstruation on food tolerance in diabetes mellitus. J. Am. med. Ass. 76: 1742-1749.
448. ROSSELIN, G., ASSAN, R., YALOW, R.S. & BERSON, S.A. (1966). Separation of antibody-bound and unbound peptide hormones labelled with iodine-131 by talcum powder and precipitated silica. Nature, Lond. 212: 355-357.
449. RUBENSTEIN, A.H., LOWY, C. & FRASER, T.R. (1967). Radioimmunoassay of insulin in urine. Diabetologia 3: 453-459.
450. RUBENSTEIN, A.H., LOWY, C., WELBORN, T.A. & FRASER, T.R. (1967). Urine insulin in normal subjects. Metabolism 16: 234-244.
451. RUBENSTEIN, A.H., POTTENGER, L.A., MAKO, M., GETZ, G.S. & STEINER, D.F. (1972). The metabolism of proinsulin and insulin by the liver. J. clin. Invest. 51: 912-921.
452. RUBIN, A. & BABBOT, D. (1958). Impotence and diabetes mellitus. J. A. med. Ass. 168: 498-500.
453. SAEZ, J.M., MOREIRA, A.M., DOZORD, A. & BERTRAND, J. (1972). Adrenal and testicular contribution to plasma oestrogens. J. Endocr. 55: 41-49.

454. SALHANICK, H.A., KIPNIS, D.M. & VANDE WIELE, R.L.
(eds) (1969). Metabolic effects of gonadal hormones and contraceptive steroids. New York & London: Plenum Press.
455. SALWAY, J.G. (1969). The simultaneous determination of metoacetate and glucose in capillary blood. Clinica chim. Acta 25: 109-116.
456. SAMOLS, E. (1965). Immunochemical aspects of insulin. In On the nature and treatment of diabetes, pp. 227-246. Eds. B.S. Leibel & G.A. Wrenshall. Amsterdam: Excerpta Medica Foundation.
457. SAMOLS, E. & BILKUS, D. (1964). A comparison of insulin immunoassays. Proc. Soc. exp. Biol. Med. 115: 79-84.
458. SAMOLS, E. & WILLIAMS, H.S. (1961). Trace-labelling of insulin with iodine. Nature, Lond. 190: 1211-1212.
459. SANDSTRÖM, B. (1969). Diabetes mellitus and menstruation. Nord. Med. 81: 727-728.
460. SCHALCH, D.S. & REICHLIN, S. (1966). Plasma growth hormone concentration in the rat determined by radio-immunoassay: influence of sex, pregnancy, lactation, anaesthesia, hypophysectomy and extracellular pituitary transplants. Endocrinology 79: 275-280.
461. SCHALLY, A.V., ARIMURA, A. & KASTIN, A.J. (1973). Hypothalamic regulatory hormones. Science, N.Y. 179: 341-350.
462. SCHOFFLING, K. (1965). Hypogonadism in male diabetic subjects. In On the nature and treatment of diabetes, pp. 505-521. Eds B.S. Leibel & G.A. Wrenshall. Amsterdam: Excerpta Medica Foundation.

463. SCHREIBEMAN, P.H. (1968). Alterations in carbohydrate and lipid metabolism by a progestin. Diabetes 17: 341-342.
464. SCHREUDER, H.B. (1972). Influence of age on insulin secretion and lipid mobilization after glucose stimulation. Israel J. med. Sci. 8: 832-833.
465. SCHWARTZ, N.B. (1969). A model for the regulation of ovulation in the rat. Recent Prog. Horm. Res. 25: 1-55.
466. SEAL, U.S. & DOE, R.P. (1969). Effects of gonadal and contraceptive hormones on protein and amino acid metabolism. In Metabolic effects of gonadal hormones and contraceptive steroids, pp. 277-318. Eds H.A. Salhanick, D.M. Kipnis & R.L. Vande Wiele. New York and London: Plenum Press.
467. SEARLE SCIENTIFIC SERVICES (1969). Pergonal: introduction and detailed dosage recommendations. High Wycombe, Bucks: G.D. Searle & Co. Ltd.
468. SERRANO-RIOS, M., HAWKINS, F.G., ESCOBAR, F., MATO, J.M., LARRODERA, L., OYA, M. & RODRIGUEZ-MINON, J.L. (1972). Insulin secretion in Klinefelters syndrome. The effect of testosterone. In Abstracts of the 8th Annual Meeting of the European Association for the Study of Diabetes. Novo Service. Abstract 246.
469. SHAIKH, A.A. (1971). Estrone and estradiol levels in the ovarian venous blood from rats during the estrous cycle and pregnancy. Biol. Reprod. 5: 297-307.
470. SHELDON, J. & TAYLOR, K.W. (1965). The immunoassay of insulin in human serum with sodium ethylene diamine tetra-acetate. J. Endocr. 33: 157-158.

471. SIEGEL, P.S. (1961). Food intake in the rat in relation to the dark-light cycle. J. comp. physiol. Psychol. 54: 294-301.
472. SILVERSTONE, F.A., BRANDFONBRENER, M., SHOCK, N.W. & YIENGST, M.J. (1957). Age differences in the intravenous glucose tolerance tests and the response to insulin. J. clin. Invest. 36: 504-514.
473. SINGH, D.J. & TURNER, C.W. (1972). Effect of melatonin upon thyroid hormone secretion rate in female hamsters and male rats. Acta endocr., Copnh. 69: 35-40.
474. SIREK, O.U. & BEST, C.H. (1953). The protein anabolic effect of testosterone propionate and its relationship to insulin. Endocrinology 52: 390-395.
475. SKELLEY, D.S., BORWN, L.P. & BESCH, P.K. (1973). Radioimmunoassay. Clin. Chem. 19: 146-186.
476. SKÖM, J.H. & TALMAGE, D.W. (1958). Nonprecipitating insulin antibodies. J. clin. Invest. 37: 783-786.
477. SLONAKER, J.R. (1924). The effect of pubescence, oestruation and menopause on the voluntary activity of the albino rat. Am. J. Physiol. 68: 294-315.
478. SLONAKER, J.R. (1925). The effect of copulation, pregnancy, pseudopregnancy and lactation on voluntary activity and food consumption of the albino rat. Am. J. Physiol. 71: 362-394.
479. SMELLIE, R.M.S. (Ed.) The biochemistry of steroid hormone action. London and New York: Academic Press.
480. SMITH, L.F. (1972). Amino acid sequences of insulins. Diabetes 21: Suppl. 2, 457-460.

481. SOELDNER, J.S., CANNON, J.J. & GLEASON, R.F. (1971). Sex differences between oral and intravenous glucose stimulated insulin release in normal and prediabetic subjects. Diabetes 20: 378-379.
482. SOELDNER, J.S. & SLONE, D. (1965). Critical variables in the radioimmunoassay of serum insulin using the double antibody technic. Diabetes 14: 771-779.
483. SONG, C.S. & KAPPAS, A. (1968). The influence of estrogens, progestins, and pregnancy on the liver. Vitams Horm. 26: 147-195.
484. SOULAIRAC, A., DESCLAUX, P. & KATZ, R.P. (1948). Action du diabète alloxanique sur le tractus genital du rat male et femelle. C. r. Séanc. Soc. Biol. 142: 311-312.
485. SPELLACY, W.N. & CARLSON, K.L. (1966). Plasma insulin and blood glucose levels in patients taking oral contraceptives. Am. J. Obstet. Gynec. 95: 474-478.
486. SPELLACY, W.N., CARLSON, K.L. & BIRK, S.A. (1967a). Carbohydrate metabolic studies after six cycles of combined type oral contraceptive tablets. Measurement of plasma insulin and blood glucose levels. Diabetes 16: 590-594.
487. SPELLACY, W.N., CARLSON, K.L. & SCHADE, S.L. (1967b). Human growth hormone (HGH) measurements in subjects taking oral contraceptives. Clin. Res. 15: 330.
488. SPELLACY, W.N., CARLSON, K.L. & SCHADE, S.L. (1967c). HGH levels in normal subjects receiving oral contraceptives. J. Am. med. Ass. 202: 451-454.
489. SPELLACY, W.N., CARLSON, K.L. & SCHADE, R.M. (1967d). Menstrual cycle carbohydrate metabolism. Am. J. Obstet. Gynec. 99: 382-386.

490. SPELLACY, W.N., CARLSON, K.L., BIRK, S.A. & SCHADE, S.L. (1968a). Glucose and insulin alterations after one year of combination-type oral contraceptive treatment. Metabolism 17: 496-501.
491. SPELLACY, W.N., BUHI, W.C., SPELLACY, C.E., MOSES, L.C. & GOLDZIEHER, J.W. (1968b). Carbohydrate studies in long-term users of oral contraceptives. Diabetes 17: (suppl.1), 344-345.
492. SPELLACY, W.N., CARLSON, K.L. & SCHADE, S.L. (1968c). Effect on a sequential oral contraceptive on plasma insulin and blood glucose levels after six months treatment. Am. J. Obstet. Gynec. 101: 672-676.
493. SPELLACY, W.N. (1969a). A review of carbohydrate metabolism and the oral contraceptives. Am. J. Obstet. Gynec. 104: 448-460.
494. SPELLACY, W.N., BENDEL, R.P., BUHI, W.C. & BIRK, S.A. (1969a). Insulin and glucose determinations after two and three years of use of a combination-type oral contraceptive. Fert. Steril. 20: 892-902.
495. SPELLACY, W.N., BUHI, W.C. & BENDEL, R.P. (1969b). Growth hormone and glucose levels after one year of combination-type oral contraceptive treatment. Int. J. Fert. 14: 51-55.
496. SPELLACY, W.N. & BUHI, W.C. (1969). Pituitary growth hormone and placental lactogen levels measured in normal term pregnancy and at early and late postpartum periods. Am. J. Obstet. Gynec. 105: 888-896.
497. SPELLACY, W.N., BUHI, W.C., SPELLACY, C.E., MOSES, L.E. & GOLDZIEHER, J.W. (1970a). Glucose, insulin and growth hormone studies in long-term users of oral contraceptives. Am. J. Obstet. Gynec. 106: 173-182.

498. SPELLACY, W.N., ZARTMAN, E.R., BUHI, W.C. & SPELLACY, C.E. (1970b). Cross-sectional investigation of carbohydrate metabolism in women taking a sequential or combination-type oral contraceptive. Southern Med. J. 63: 152-155.
499. SPELLACY, W.N. (1971). Plasma insulin, growth hormone and placental lactogen levels in normal and abnormal pregnancies. Acta endocr., Copnh. 67: (suppl.155), 82.
500. SPELLACY, W.N. & BUHI, W.C. (1971). Serum-insulin or plasma-insulin? Lancet i: 87.
501. SPELLACY, W.N., BUHI, W.C., BIRK, S.A. & McCREARY, S.A. (1971a). Studies of ethynodiol diacetate and mestranol on blood glucose and plasma insulin. Int. J. Fert. 16: 55-65.
502. SPELLACY, W.N., BUHI, W.C., BIRK, S.A. & McCREARY, S.A. (1971b). Studies of ethynodiol diacetate and mestranol on blood glucose and plasma insulin. II. Twelve month OGTT. Contraception 3: 185-194.
503. SPELLACY, W.N., BUHI, W.C., BIRK, S.A. & McCREARY, S.A. (1971c). Studies of chlormadinone acetate and mestranol on blood glucose and plasma insulin. I. Six month OGTT. Fert. Steril. 22: 217-223.
504. SPELLACY, W.N., BUHI, W.C.; BIRK, S.A. & McCREARY, S.A. (1971d). Studies of chlormadinone acetate and mestranol on blood glucose and plasma insulin. II. Twelfth month OGTT. Fert. Steril. 22: 224-228.
505. SPELLACY, W.N., BUHI, W.C. & BIRK, S.A. (1972a). The effect of the progestogen ethynodiol diacetate on glucose, insulin and growth hormone after six months treatment. Acta endocr., Copnh. 70: 373-384.

506. SPELLACY, W.N., McLEOD, A.G.W., BUHI, W.C. & BIRK, S.A. (1972b). The effects of medroxyprogesterone acetate on carbohydrate metabolism: measurements of glucose, insulin and growth hormone after 12 months use. Fert. Steril. 23: 239-244.
507. SPELLACY, W.N., BUHI, W.C. & BIRK, S.A. (1972c). The effect of estrogens on carbohydrate metabolism: glucose, insulin and growth hormone studies on one hundred and seventy-one women ingesting Premarin, mestranol, and ethinyl estradiol for six months. Am. J. Obstet. Gynec. 114: 378-392.
508. SPELLACY, W.N. & GOETZ, F.C. (1963). Plasma insulin in normal late pregnancy. New Engl. J. Med. 268: 988-991.
509. SPIEGELMAN, A.R. (1940). Influence of estrogen on the insulin requirement of the diabetic. Am. J. med. Sci. 200: 228-234.
510. SPRINGELL, P.H. (1961). An unreactive tyrosine residue in insulin and the exclusive iodination of the A chain. Nature, Lond. 191: 1372-1373.
511. STARUP, J., DALE, J. & DECKERT, T. (1968). Serum insulin and intravenous glucose tolerance in oral contraception. Acta endocr., Copnh. 58: 537-544.
512. STEINER, D.F., HALLUND, O., RUBENSTEIN, H., CHO, S. & BAYLISS, C. (1968). Isolation and properties of proinsulin, intermediate forms, and other minor components from crystalline bovine insulin. Diabetes 17: 725-736.
513. STEINER, D.F., KEMMLER, W., CLARK, J.L., OYER, P.E. & RUBENSTEIN, A.H. (1972). The biosynthesis of insulin. In Handbook of physiology, section 7, vol.1, pp. 175-198. Eds R.O. Greep & E.B. Astwood. Washington, D.C.: American Physiological Society.

514. STERN, J.S. & HIRSCH, J. (1972). Obesity and pancreatic function. In Handbook of physiology, section 7, vol.1, pp. 641-651. Eds R.O. Greep & E.B. Astwood. Washington, D.C.: American Physiological Society.
515. STEWART, G.A. (1960). Methods of insulin assay. Br. med. Bull. 16: 196-201.
516. STONE, S.H. (1954). Method for obtaining venous blood from the orbital sinus of the rat or mouse. Science, N.Y., 119: 100.
517. SUN, A.M., LIN, B.J. & HAIST, R.E. (1972). Studies on the effects of growth hormone and thyroxine on proinsulin synthesis and insulin formation in the isolated islets of Langerhans of the rat. Can. J. Physiol. Pharmacol. 50: 1147-1151.
518. SUSSMAN, K.E., VAUGHAN, G.D. & TIMMER, R.F. (1966). An invitro method for studying insulin secretion in the perfused isolated rat pancreas. Metabolism 15: 466-476.
519. SUTTER-DUB, M.-Th., LECLERCQ, R., FELIX, J.M., JACQUOT, R. & SUTTER, B.Ch.J. (1973). Serum progesterone and immunoreactive insulin levels in the pregnant rat. Horm. Metab. Res. 5: 18-21.
520. SWERDLOFF, R.S., JACOBS, H.S. & ODELL, W.D. (1972). Hypothalamic-pituitary-gonadal interrelationships in the rat during sexual maturation. In Gonadotropins, pp. 546-561. Eds B.B. Saxena, C.G. Beling & H.M. Gandy. New York: Wiley-Interscience.
521. SZABO, A.J., COLE, H.S. & GRIMALDI, R.D. (1970). Glucose tolerance in gestational diabetic women during and after treatment with a combination-type oral contraceptive. New Engl. J. Med. 282: 646-650.

522. SZABO, O. & SZABO, A.J. (1972). Evidence for an insulin-sensitive receptor in the central nervous system. Am. J. Physiol. 223: 1349-1353.
523. TAFT, P., WINIKOFF, D., TAYLOR, K. PAGE, D. & BRITTINGHAM, L.C. (1969). The influence of oral contraceptives with and without an ovulation suppressant action on thyroid function tests. Preliminary observations on differences in insulin response to glucose. In Chlormadinoneacetate - a new departure in oral contraception, pp. 60-72. Amsterdam: Excerpta Medica Foundation.
524. TALAAT, M., HABIB, Y. & HABIB, M. (1957). The effect of testosterone on carbohydrate metabolism in normal subjects. Archs int. Pharmacodyn. Ther. III: 215-226.
525. TALAAT, M., HABIB, Y., HAMDI, H. & MALEK, A.Y. (1965). Effect of oestradiol dipropionate on the carbohydrate metabolism in castrated male rabbits. Archs int. Pharmacodyn. Ther. 154: 235-242.
526. TALAAT, M., HABIB, Y. & HANNA, T. (1958). Effect of testosterone propionate on carbohydrate metabolism in the liver and muscle of male rabbits. Archs int. Pharmacodyn. Ther. 116: 410-417.
527. TALAAT, M., HABIB, Y., HIGAZY, A.M., NABY, S.A., MALEK, A.Y. & IBRAHIM, Z.A. (1965a). Effect of sex hormones on the carbohydrate metabolism in normal and diabetic women. Archs int. Pharmacodyn. Ther. 154: 402-411.
528. TALAAT, M., HABIB, Y.A. & MALEK, A. (1960). Effect of oestradiol dipropionate on carbohydrate metabolism in the liver and muscle of adult female rabbits. Archs int. Pharmacodyn. Ther. 123: 490-497.

529. TALAAT, M., HABIB, Y. & MALEK, A. (1964). Effect of testosterone propionate on the carbohydrate metabolism in castrated male rabbits. Archs int. Pharmacodyn. Ther. 151: 369-382.
530. TÄLJEDAL, I.B. & WOLD, S. (1970). Fit of some analytical functions to insulin radio-immunoassay standard curves. Biochem. J. 119: 139-143.
531. TARTTELIN, M.F. & GORSKI, R.A. (1971). Variations in food and water intake in the normal and acyclic female rat. Physiol. behav. 7: 847-852.
532. TAYLOR, K.W. (1967). Insulin. In Hormones in blood, 2nd edn, vol.1, pp. 47-81. Eds C.H. Gray & A.L. Bacharach. London and New York: Academic Press.
533. TAYLOR, K.W., HOWELL, S.L., MONTAGUE, W. & EDWARDS, J.C. (1968). Immunoassay of insulin and glucagon. Clinica chim. Acta 22: 71-76.
534. TAYLOR, M.B. & KASS, M.B. (1968). Effect of oral contraceptives on glucose metabolism. Am. J. Obstet. Gynec. 102: 1035-1038.
535. TEJNING, S. (1947). Dietary factors and quantitative morphology of the islets of Langerhans. Acta med. scand. 128: (suppl.198), 1-154.
536. TEPPERMAN, J. (1968). Metabolic and endocrine physiology, 2nd edn. Chicago: Year Book Medical Publishers Incorporated.
537. TERPSTRA, P., REITSMA, W.D., VISSER, J.W.E. & DOORENBOS, H. (1970). The influence of an oral contraceptive on glucose-, F.F.A.-, triglyceride- and insulin levels after glucose loading. Folia Med. Neerl. 13: 44-50.

538. THOMAS, P.J. (1973). Steroid hormones and their receptors. J. Endocr. 57: 333-359.
539. THORELL, J.I. & LANNER, A. (1973). Influence of heparin-plasma; EDTA-plasma, and serum on the determination of insulin with three different radio-immunoassays. Scand. J. clin. Lab. Invest. 31: 187-190.
540. TOBIN, J.D., ANDRES, R., SHERWIN, R., LILJENQUIST, J. & WEISSMAN, P. (1970). The mechanism of the decline in glucose tolerance with age: decreased insulin release or decreased sensitivity to insulin? In Abstracts of the 7th Congress of the International Diabetes Federation, p. 141. Int. Congr. Series no.209. Amsterdam: Excerpta Medica Foundation.
541. TRAUTSCHOLD, E., WERLE, E. & ZICKGRAF-RUDEL, G. (1967). Trasylol. Biochem. Pharmac. 16: 59-72.
542. TRAYNER, I.M., WELBORN, T.A., RUBENSTEIN, A.H. & RUSSEL-FRASER, T. (1967). Serum and urine insulin in late pregnancy and in a few pregnant latent diabetics. J. Endocr. 37: 443-453.
543. UCHIDA, K., KADOWAKI, M. & MIYAKE, T. (1969). Ovarian secretion of progesterone and 20 α -hydroxypregn-4-en-3-one during rat estrous cycle in chronological relation to pituitary release of luteinizing hormone. Endocr. jap. 16: 227-237.
544. UNITED STATES DEPARTMENT OF HEALTH, EDUCATION AND WELFARE (1966). Childbearing and diabetes mellitus. U.S. Public Health Service, series 11, number 21: 1-19.
545. UNIVERSITY OF ASTON (1969). Recommendations and notes for the guidance of departments working with radioactive substances and ionising radiations. Birmingham: Health Centre, University of Aston.

546. URSELL, W., BRUDENELL, M. & CHARD, J. (1973).
Placental lactogen levels in diabetic pregnancy.
Br. med. J. 2: 80-82.
547. VALLANCE-OWEN, J. & WRIGHT, P.H. (1960). Assay of
insulin in blood. Physiol. Rev. 40: 219-244.
548. VARADANI, P.T. (1968). Plasma concentrations of A and
B chains of insulin in nondiabetic, diabetic and high
risk potential diabetic subjects. Diabetes 17:
547-556.
549. VELASCO, C.A., OPPERMANN, W. & CAMERINI-DAVALOS, R.A..
(1973). Critical variables in the radioimmunological
technique for measuring immunoreactive insulin with
use of immunosorbents. Clin. Chem. 19: 201-204.
550. VERMEULEN, A., DANEELS, R. & THIERY, M. (1970). Effects
of oral contraceptives on carbohydrate metabolism.
Diabetologia 6: 519-523.
551. VILLAR-PALASI, C. (1968). The hormonal regulation of
glycogen metabolism in muscle. Vitams Horm. 26:
65-118.
552. VON NOORDEN, C.H. & ISAAK, S. (1927). Die Zucker-
krankheit und ihre Behandlung, 8th edn. Berlin:
J. Springer.
553. WADE, G.N. (1972). Gonadal hormones and behavioural
regulation of body weight. Physiol. Behav. 8:
523-534.
554. WAINE, H., FRIEDEN, E.H., CAPLAN, H.I. & COLE, T.
(1963). Metabolic effects of Enovid in rheumatoid
patients. Arthr. Rheum. 6: 796.

555. WALDHAUSL, W., BERINGER, A. & SCHNEIDER, W. (1969). Die Beeinflussung der Glukosetoleranz und der Plasminsulinkonzentration durch Ovulationshemmer. Geburtsh Frauenheilk 29: 611-617.
556. WANG, G.H. (1923). The relation between the "spontaneous" activity and the oestrous cycle in the white rat. Comp. Psychol. Monogr. 2: 1-27.
557. WARREN, J.C. (1971). Estrogens and uterine metabolism. In The sex steroids, pp. 149-180. Ed. K.W. McKerns. New York: Meredith Corporation.
558. WARREN, M.P. (1973). Metabolic effects of contraceptive steroids. Am. J. med. Sci. 265: 5-21.
559. WELBORN, T.A. & FRASER, T.R. (1965). The double-antibody immunoassay of insulin. A standardized second antibody reaction that eliminates spurious results with human serum. Diabetologia 1: 211-218.
560. WELBORN, T.A., RUBENSTEIN, A.H., HASLAM, R. & FRASER, R. (1966). Normal insulin response to glucose. Lancet 1: 280-284.
561. WELBORN, T.A., STENHOUSE, N.S., CURNOW, D.H. & JOHNSTONE, C.J. (1970). Double antibody method for immunoassay of insulin. Its application in population studies. In In vitro procedures with radioisotopes in medicine, pp. 525-536. Vienna: International Atomic Energy Agency.
562. WELLCOME RESEARCH LABORATORIES (1973). Personal communication, Wellcome Research Laboratories, Beckenham.
563. WIDE, L., AXEN, R. & PORATH, J. (1967). Radio-immunoabsorbent assay for proteins. Chemical coupling antibodies to insoluble dextran. Immunochem. 4: 381-386.

564. WILLIAMS, R.H. (editor) (1969). Textbook of endocrinology. Philadelphia, London and Toronto: W.B. Saunders Company.
565. WILLIAMS-ASHMAN, H.G. & REDDI, A.H. (1971). Actions of vertebrate sex hormones. A. Rev. Physiol. 33: 31-82.
566. WILSON, J.D. (1972). Recent studies on the mechanism of action of testosterone. New Engl. J. Med. 287: 1284-1291.
567. WOLDOW, A., SHAPIRO, B., COHEN, J.J. & KOLLMAN, G. (1972). Relationship of lipids to glucose tolerance and insulin response in the aged. J. Am. ger. Soc. 20: 515-520.
568. WRIGHT, B.A. (1970). A new device for collecting blood from rats. Lab. Anim. Care 20: 274-275.
569. WRIGHT, P.H. (1965). Hormone antibodies in endocrinology. Vitams Horm. 23: 61-95.
570. WRIGHT, P.H., KREISBERG, R.A., HALPERN, B. & DOLKART, R.E. (1962). Properties of insulin antibodies produced by the guinea pig, horse, sheep and man. Diabetes 11: 519-526.
571. WRIGHT, P.H., MAKULU, D.R., MALAISSE, W.J., ROBERTS, N.M. & YU, P. (1968). A method for the immunoassay of insulin. Diabetes 17: 537-546.
572. WRIGHT, P.H., MAKULU, D.R., VICHICK, D. & SUSSMAN, K.E. (1971). Insulin immunoassay by back-titration; some characteristics of the technic and the insulin precipitant action of alcohol. Diabetes 20: 33-45.

573. WRIGHT, W.R., RAINWATER, J.C. & TOLLE, L.D. (1971).
Glucose assay systems: evaluation of a colorimetric
hexokinase procedure. Clin. Chem. 17: 1010-1015.
574. WURTMAN, R.J. (1969). The pineal gland in relation
to reproduction. Am. J. Obstet. Gynec. 104: 320-326.
575. WURTMAN, R.J., AXELROD, J. & KELLY, D.E. (1968).
The pineal. New York: Academic Press.
576. WURTMAN, R.J., AXELROD, J., SYNDER, S.H. & CHU, E.W.
(1965). Changes in the enzymatic synthesis of
melatonin in the pineal during the estrous cycle.
Endocrinology 76: 798-800.
577. WYNN, V. & DOAR, J.W.H. (1966). Some effects of oral
contraceptives on carbohydrate metabolism. Lancet 2:
715-719.
578. WYNN, V. & DOAR, J.W.H. (1969). Longitudinal studies
on the effects of oral contraceptive therapy in plasma
glucose, non-esterified fatty acid, insulin and blood
pyruvate levels during oral and intravenous glucose
tolerance tests. In Metabolic effects of gonadal
hormones and contraceptive steroids, pp. 157-177.
Eds H.A. Salhanick, D.M. Kipnis & R.L. Vande Wiele.
New York & London: Plenum Press.
579. YALOW, R.S. & BERSON, S.A. (1960). Immunoassay of
endogenous plasma insulin in man. J. clin. Invest. 39:
1157-1175.
580. YALOW, R.S. & BERSON, S.A. (1968). General principles
of radioimmunoassay. In Radioisotopes in medicine:
in vitro studies, pp. 7-41. Eds R.L. Hayes,
F.A. Goswitz & B.E.P. Murphy. AEC Symp. Series no.13.
Oak Ridge, Tennessee: Atomic Energy Commission.

581. YEN, S.S.C., TSAI, C.C. & VELA, P. (1971).
Gestational diabetogenesis: quantitative analyses
of glucose-insulin relationship between normal
pregnancy and pregnancy with gestational diabetes.
Am. J. Obstet. Gynec. 111: 792-800.
582. YEN, S.S.C. & VELA, P. (1968). The effects of
contraceptive steroids on carbohydrate metabolism.
J. clin. Endocr. Metab. 28: 1564-1570.
583. YEN, S.S.C. & VELA, P. (1969). Carbohydrate metabolism
and long-term use of oral contraceptives. J. Reprod.
Med. 3: 6-18.
584. YUDAKEN, I.R. & BLOOMBERG, B.M. (1973). Oral glucose
tolerance tests. S. Afr. Med. J. 47: 865-867.
585. YOSHINAGA, K., HAWKINS, R.A. & STOCKER, J.F. (1969).
Estrogen secretion by the rat ovary in vivo during
the estrous cycle and pregnancy. Endocrinology 85:
103-112.
586. ZAHARKO, D.S. & BECK, L.V. (1968). Studies of a
simplified plasma insulin immunoassay using cellulose
powder. Diabetes 17: 444-457.
587. ZARROW, M.X., YOCHIM, J.M. & MCCARTHY, J.L. (1964).
Experimental endocrinology: a sourcebook of basic
techniques. New York and London: Academic Press.
588. ZEYTINOGLU, I.Y., GHERONDACHE, C.N. & PINCUS, G.
(1969). The process of aging: serum glucose and
immunoreactive insulin levels during the oral glucose
tolerance test. J. Am. ger. Soc. 17: 1-14.
589. ZEYTINOGLU, I.Y. & GHERONDACHE, C.N. (1970). Serum
immunoreactive insulin (IRI) responses to oral glucose
tolerance tests (OGTT) in the elderly. In Abstracts
of the 7th Congress of the International Diabetes
Federation, pp. 24-25. Int. Congr. Series no.209.
Amsterdam: Excerpta Medica Foundation.